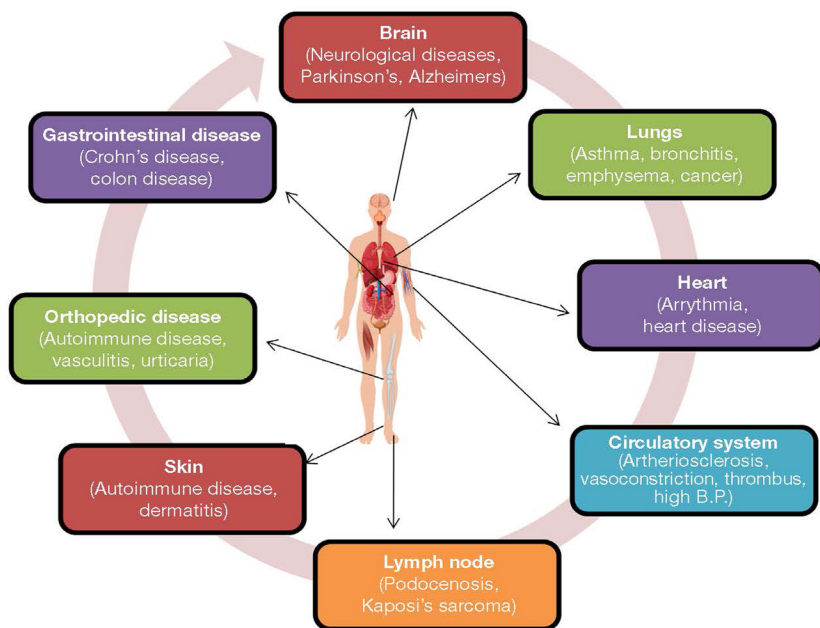


Nanotoxicology

Toxicity Evaluation of Nanomedicine Applications

Edited by
Hemant Kumar Daima
S.L. Kothari
Suresh Kumar Bhargava



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*This book is dedicated to my little son Yuvaan, who has
brought joy, happiness, and childhood to my life*

*Dearest Sister Urmila, while you are in a better
place and in my thoughts, I wish you were around
to eyewitness my accomplishments.*

*Dearest Dad, Mom, Kalpana, Lokesh, Aarti, Daksh,
and Tashu, the journey of this book would not have been
possible without your love, support, patience, and sacrifices.
Thank you for being the pillars of my strength.*

Hemant Kumar Daima

To my family and students.

S. L. Kothari

To my mother, teachers, mentors and my all students.

Suresh Kumar Bhargava



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Preface

“Nanotechnology—The Biggest Discovery of 21st Century and an Invisible Hope for Tomorrow”

Hemant Kumar Daima, S. L. Kothari and Suresh Kumar Bhargava

The field of nanomedicine has emerged rapidly due to myriad designer-made nanomaterials. The nanomaterials possess significant potential to manage diseases, and they can change the face of medicine, as we see today. However, the amplified practices of nanomaterials, their release into the environment, and their contact with living entities have raised serious threats. It has also been realized that many of the important concepts of nanomedicine and nanotoxicity have been overlooked, and they must be attended promptly. Therefore, this book takes a systematic approach to address the gaps relating to nanomedicine and nanotoxicity and bring together fragmented knowledge on the advances toward safe-by-design nanomaterials and existing toxicity challenges.

Nanotoxicology: Toxicity Evaluation of Nanomedicine Applications demonstrates an exclusive compilation of state of the art with a focus on fundamental concepts of nanomedicine and nanotoxicity, current trends, limitations, regulatory challenges, nano-ethics, and future directions of toxicity evaluation of nanomaterials. First, this book discusses the fundamental aspects of various nanomaterials along with their important physicochemical properties. The physicochemical properties of nanomaterials are important to translate their therapeutic or potential toxic influence. A few chapters deliberate on the interfacial interactions, their importance in clinical problems and applications in sensing, imaging, drug/gene delivery, tissue engineering, and medical devices. Later, toxicity of nanomaterials, the mechanisms of nanotoxicity, and its impact on environment and health are discussed. Nevertheless, the functional toxicity assessment of nanomaterials is difficult. Therefore, we bring together the available innovative approaches to assess the toxicity of nanomaterials along with *in vitro* and *in vivo* test methods in some chapters. This is followed by the recommendations on nanomaterials' toxicity and safe-by-design nanomedicines. At the end of this book, the chapters are devoted to assorted challenges in the clinical translation of nanomedicines, wherein debates on the regulatory challenges, legal, political, societal, and ethical issues of nanomaterials along with recommendations for future work in the area are provided.

In *Nanotoxicology: Toxicity Evaluation of Nanomedicine Applications*, diverse groups of internationally recognized experts discuss their insights on Nanomedicine and Nanotoxicology to make it versatile. This book is aimed toward postgraduates, academics, engineers, scientists, industry professionals, and policy makers. This book contains 17 chapters, and it has noteworthy importance for excellent foundation due to its unique A to Z aspect.



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The editors acknowledge all the authors and express gratitude for accepting the invitation to contribute to this book. It is their commitment and involvement which has brought sporadic knowledge to systematic chapters. Further, the editors are thankful to all the reviewers who have offered valuable suggestions to enhance the quality of the chapters.

The editors further admire the CRC Press team for considerable assistance and cooperation to produce this important book.

At the end, the editors generously acknowledge the almighty “God” and members of their families who have provided the motivation and strength to work on this book from the conception of the idea.

Hemant Kumar Daima, Jaipur, Rajasthan, India
S. L. Kothari, Jaipur, Rajasthan, India
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Editors



Hemant Kumar Daima, PhD, MRSC, is an Indian scientist, academician, and administrator. He is a Professor at Amity University Rajasthan, India; in-charge of Amity University Science & Instrumentation Center-II (AUSIC-II); founding member and coordinator of Amity Center for Nanobiotechnology and Nanomedicine (ACNN); and “honorary visiting scientist” at RMIT University, Australia.

Dr. Daima has expertise in designing nanoparticles with controlled physicochemical properties by employing green chemistry routes. He has demonstrated the importance of surface functionalization of nanomaterials to control corona, which dictates nanomaterials’ interaction at nano-bio interface. His research findings have revealed guiding principles involved in rational nanoparticle design strategies for biomedical applications. Currently, Dr. Daima’s team is working on the development of functional organic and inorganic nanomaterials for drug/gene delivery, biosensors, management of multi-drug resistant (MDR) bacteria, nanozyme activities, and medical devices.

Dr. Daima is editorial board member and reviewer of leading international publishers in the field of Nanotechnology, Nanotoxicology, and Nanomedicine, with more than 50 peer-reviewed, high-impact publications to date. Dr. Daima has presented his research worldwide, and he is member of several scientific/professional bodies. He is recipient of numerous international fellowships/awards, and he has established Nano-Bio Interfacial Research Laboratory (NBIRL) to undertake high-quality fundamental and applied research. He obtained MSc (Biotechnology) from University of Rajasthan, India and PhD (Nanobiotechnology) under the supervision of Distinguished Professor Suresh Kumar Bhargava from RMIT University, Australia.



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Prof. Kothari graduated from the University of Rajasthan, Jaipur, India in 1984 with a PhD in Biotechnology, and during his long career, he held post-doctoral positions as Fulbright Fellow at the University of Illinois, Urbana-Champaign, USA (1983–1984); as Commonwealth Academic

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Suresh Kumar Bhargava, PhD in Chemistry (University of Exeter, UK), **DSc, FAAS, FTSE, FNAE, FNASI, FRSC, FRACI**, is a world leader in the field of advanced materials, particularly his landmark discoveries to explore properties of gold at both molecular and nano-level for sensing, catalysis, and biomedical applications are now well established. He is now a leading metallo-drug scientist in Australia. His innovation-driven research has led to creating critical scientific knowledge and delivering outstanding engineering and scientific solutions. The impact

of this cohesive body of contribution is attested by his highly cited refereed journal articles (530), book chapters (16), plenary/keynote lecturers (>280), patents (>10), and numerous accolades with a total citation more than 17300. His success arises from his unique ability to conduct cutting-edge research and translate the outcomes for significant national and global impact, mentor young scientists to realize their potential, facilitate intellectual synergy by leading large multidisciplinary and multinational teams, and bridge the gap between pure and applied research by crossing disciplines boundaries for real-world applications. He has supervised more than 60 PhD joining his group all over the world.

He has been recognized through many prestigious national and international awards including the 2017 TIMES Network NRI of the Year Award, the 2016 Khwarizmi International Award (KIA) by the Government of Iran, the 2015 CHEMECA medal, the most prestigious Indian National Science Academy's P. C. Ray Chair (distinguished lecture series 2014), and RMIT University Vice Chancellor's Research Excellence Award twice (2006 and 2014). He is an elected fellow of seven learned academies around the world and has been visiting distinguished professor at many universities around the globe including, IISc, University of Tokyo, International University of Florida, Rajasthan University, Jaipur, IIT Bombay and the University of Malaya, Malaysia.

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1 Nanomaterials

Types of Nanomaterials and Their Fundamental Physicochemical Properties

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1.1 INTRODUCTION TO NANOMATERIALS

Nanomaterials are the materials having at least one of the dimensions in nanometer scale, i.e. in the range of 1–100 nm. The word “nano” originates from the Greek word “nanos” (or Latin “nanus”) which means “dwarf,” i.e. an abnormally short person. However, scientifically, the word “nano” is a prefix which is equal to 10^{-9} or one billionth. Hence, one nanometer is equal to one billionth of a meter ($1 \text{ nm} = 10^{-9} \text{ m}$). The following examples help to understand a sense of nanoscaled objects: (i) diameter of a hydrogen atom is about 0.1 nm (if ten hydrogen atoms are aligned in a line, then the resulting length would be approximately 1 nm), (ii) diameter of single strand of human hair is about 20,000 nm, and (iii) size of a DNA molecule is about 2.5 nm.

The nanomaterials are not new, but only the term “nanomaterial” used to name these materials is new. Human beings have used these materials for centuries without knowing that they are nanomaterials. The nanomaterials were in existence from the Roman period. Romans used colloidal metals to treat arthritis, to dye fabrics, and to color glasses.

The Purple of Cassius, a popular dye made up of tin oxide and gold nanoparticles, can be obtained by reacting stannic acid with chloroauric acid. Romans were experts in impregnating glasses with metal particles to produce dramatic effects. The Lycurgus cup is an outstanding example for this [1].¹ The cup appears red for the transmitted light, if a light source is kept inside the cup, whereas the cup appears opaque-green for the reflected light, if the light source is kept outside the cup (Figure 1.1). These unique optical properties are due to small amounts of colloidal gold and silver present in the cup.

Michael Faraday was the first to prepare nanoparticles in laboratory. Faraday called these particles as “divided state of gold” in his diary dated April 2, 1856 [2]. His gold colloidal samples are still at the British Museum, United Kingdom showing beautiful magenta-red color but not yellow color (Figure 1.2).

The stained glass windows of medieval churches were made of gold and silver particles. Maya blue used by the Mayas consisted of silica, indigo, metal, and oxide nanocrystals. Over 5,000 years ago, the Egyptians used gold nanoparticles in dentistry. Alchemists in Alexandria had developed a powerful colloidal elixir known as “liquid gold” to restore youthfulness. The colloidal gold has been incorporated in vases and glasses for coloring them.

¹ An episode from the myth of Lycurgus, a king of the Thracians (around 800 BC), is the theme of the scene in the cup. Lycurgus attacked to kill Ambrosia, the follower of the god Dionysus. Ambrosia appealed Dionysus for help. Dionysus turned Ambrosia into vine and held Lycurgus by making Ambrosia coiling around the cruel king.



FIGURE 1.1 Lycurgus cup (An episode from the myth of Lycurgus, a king of the Thracians (around 800 BC), is the theme of the scene in the cup. Lycurgus attacked to kill Ambrosia, the follower of the god Dionysus. Ambrosia appealed Dionysus for help. Dionysus turned Ambrosia into vine and held Lycurgus by making Ambrosia coiling around the cruel king). (See the ebook for the color version of this figure.) (From https://www.britishmuseum.org/research/collection_online/collection_object_details/collection_image_gallery.aspx?partid=1&assetid=1066672&objectid=61219. With permission.)



FIGURE 1.2 Michael Faraday's colloidal gold. (See the ebook for the color version of this figure.) (From <http://personal.bgsu.edu/~nberg/faraday/diary2.htm>. With permission.)

1.1.1 NANOSCALE SIZE EFFECT

The laws of physics operate somewhat differently at nanometer length scale dimensions. At nanoscale, laws of quantum mechanics take the place of classical mechanics which are applicable for macroscopic objects. For example, at nanoscale, the surface of a table spoon is not smooth; instead, it consists of discrete atoms and/or molecules.

In general, at micrometer scale, the physical properties of materials are the same as those of their bulk counterparts. However, at nanometer scale, the materials may display physical properties different from those of their bulk counterparts. The properties of nanomaterials are different from their bulk forms due to increased surface area to volume ratio and quantum confinement effect. These concepts will be discussed in the following sections.

1.1.1.1 Surface Area and Surface Area to Volume Ratio

The properties of a material depend partly on its size. In general, the physical, chemical, and biological properties of a material differ at the nanoscale, i.e. nanomaterials, when compared to its bulk counterpart. This is due to the fact that the nanomaterials have relatively larger surface area to volume ratio than the same quantity of materials produced in a bulk form. This will make materials more chemically reactive. In several cases, materials that are inert in their bulk form become reactive in their nanoscale form, and thus, the size of materials affects their strength or electrical properties.

Let us now understand how reduction in the size of a material increases its surface area and surface area to volume ratio. Consider a cube of salt. Let us assume that the cube has sides of 2 cm length. The cube has six squared faces each having a surface area of 4 cm^2 ($=2\text{ cm} \times 2\text{ cm}$), and hence, the total surface area (S) of the cube is 24 cm^2 ($=6 \times 4\text{ cm}^2$). The volume of the cube is equal to length $\times$ breadth $\times$ height. So, the total volume (V) of the cube is 8 cm^3 ($=2\text{ cm} \times 2\text{ cm} \times 2\text{ cm}$). Hence, the S/V ratio is $3 \times 10^2\text{ m}^{-1}$ ($=24\text{ cm}^2/8\text{ cm}^3$). Similarly, for a cube with a side length of 1 cm, the S/V ratio is $6 \times 10^2\text{ m}^{-1}$ ($=6\text{ cm}^2/1\text{ cm}^3$). Similarly, for a cube with a side length of 1 nm, the S/V ratio is $6 \times 10^9\text{ m}^{-1}$ ($=6\text{ nm}^2/1\text{ nm}^3$). This illustration demonstrates that a smaller cube will have a greater surface area to volume ratio than a bigger cube.

Let the cube having 2 cm side length be divided into eight small cubes as shown in Figure 1.3. Here, each of the eight small cubes will have a side length of 1 cm, a surface area of 6 cm^2 , and a volume of 1 cm^3 . Hence, the total surface area of the eight cubes becomes 48 cm^2 ($=8 \times 6\text{ cm}^2$). If each of the 1 cm side length cubes is further divided into 10^{21} cubes, then the side length of the resulting cubes becomes equal to 1 nm. Therefore, the total surface area of 10^{21} cubes becomes $6 \times 10^7\text{ cm}^2$ ($=10^{21}\text{ cubes} \times 6\text{ faces} \times 1\text{ nm}^2$). Hence, the surface area of the 10^{21} nanometer scale cubes is about 10^6 times greater than the centimeter scale cube. This example illustrates that if the size of the particles becomes smaller and smaller, then their surface area will increase. This larger surface area permits simultaneous interaction of chemicals with catalysts, which makes

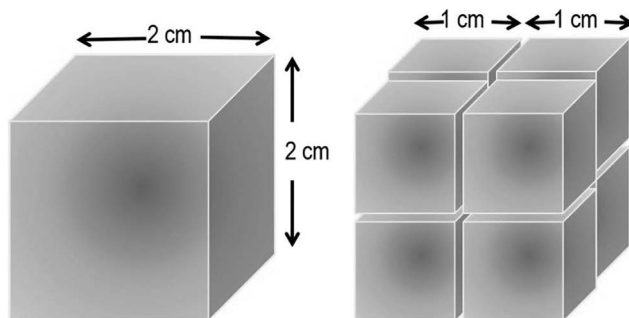


FIGURE 1.3 A cube having 2 cm side length divided into eight small cubes.

the catalysts more effective. For example, the bulk gold which is chemically inert at macroscopic scale becomes reactive and catalytic at nanoscale, and even melts at lower temperatures.

1.1.1.2 Quantum Confinement Effect

The quantum confinement is a phenomenon in which there will be a change in optical and electrical properties of materials when their size is reduced to nanometer scale (~ 100 nm or less). The “quantum size effect” is one of the examples where the electrical properties of solids undergo change due to the great reduction in the particle size. At both macroscopic and microscopic scales, this effect does not come into play and will be pronounced when the nanometer size range is reached.

In general, the quantum confinement is observed when the dimension of the material is of the order of the de Broglie wavelength of the electron involved. The confinement of the electron means restricting its movement in a space within dimensions equal to its de Broglie wavelength. The quantization of energy and momentum is a consequence of this confinement in space. Here, the principles of quantum mechanics will play an important role instead of the laws classical mechanics. The allowed energy levels can be obtained using the laws of quantum mechanics, i.e. by solving the Schrödinger wave equation with relevant boundary conditions.

Based on the direction of confinement, the quantum confined structures can be classified into three categories, namely, quantum well, quantum wire, and quantum dots. The basic quantum confined structures with quantum confinement and the number of degrees of freedom of charge carriers are shown in Table 1.1.

In a quantum dot, the charge carriers (e.g. electrons and holes) are confined in all the three directions, i.e. not free to move in any of the three directions. In a quantum wire, the charge carriers are confined in two directions and free to move in the other direction. In a quantum well, the charge carriers are confined in one direction and free to move in other two directions. In bulk materials, the charge carriers are not confined in any direction and free to move in all three directions. The number of discrete energy levels increases with the number of dimensions

TABLE 1.1
The Confinement of Quantum-Confined Structures

Structure	Quantum Confinement	Number of Degrees of Freedom of Charge Carriers
Quantum dot (0-D)	3	0
Quantum wire (1-D)	2	1
Quantum well (2-D)	1	2
Bulk (3-D)	0	3

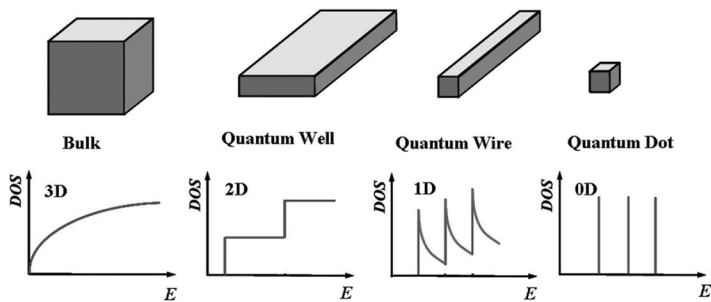


FIGURE 1.4 The schematic representation of bulk (3-D), quantum well (2-D), quantum wire (1-D), and quantum dot (0-D) structures and variation of their density of states (DOS) with energy.

confined. The schematic representation of 3-D (bulk), 2-D (quantum well), 1-D (quantum wire), and 0-D (quantum dot) semiconductor structures and their density of states (DOS)² is given in Figure 1.4.

If the size (i.e. diameter) of the particle is of the order of the de Broglie wavelength of the electron wave function, then it experiences a quantum confinement effect. The quantum confinement increases the energy difference between the energy states and band gap in the material compared to its bulk counterpart (Figure 1.5). These changes strongly affect optical and electrical properties of the material at nanoscale. If the dimensions of the confining structure are very large when compared to its de Broglie wavelength, then it behaves like a free particle. Here, the energy states are continuous and the band gap will be at its original position.

The study of the energy levels of a particle in a potential well of infinite height reveals that the spacing between the adjacent energy levels, ΔE_g , is given by Eq. (1.1).

$$\Delta E_g \propto \frac{1}{a^2} \tag{1.1}$$

² Density of states (DOS) gives the number of energy states in a given energy range.

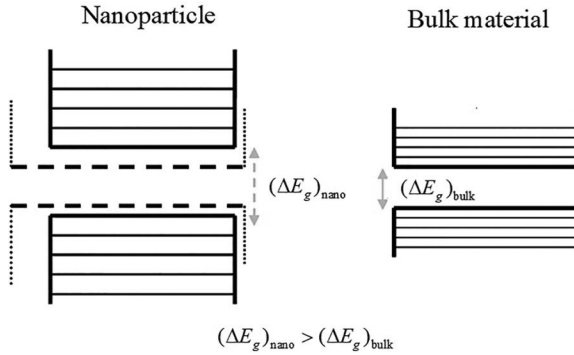


FIGURE 1.5 The increase of energy difference between energy states and band gap in a nanoparticle compared to its bulk counterpart due to the quantum confinement, where $(\Delta E_g)_{\text{nano}}$ and $(\Delta E_g)_{\text{bulk}}$ are band gap in nanoparticle and bulk material, respectively.

where “a” is the width of the potential well which should be compared with the size of the particle. Eq. (1.1) is an extremely important relation that has been experimentally well verified. It can be clearly seen that ΔE_g increases as “a” decreases. Eq. (1.1) shows that the energy difference between adjacent energy levels increases significantly with decrease in the size of the particle. This equation has a practical significance, which depicts that it is possible to tune the optical properties of the particle by varying its size, i.e. diameter. By decreasing the particle size, ΔE_g can be increased, which results in blue shift of the absorption or emission of photons.

1.1.2 CLASSIFICATION OF NANOMATERIALS

Nanomaterials can have nanometer scale in one dimension (e.g. surface films), two dimensions (e.g. strands or fibers), or three dimensions (e.g. particles). They may exist in single, fused, agglomerated, or aggregated forms with spherical, tubular, and irregular shapes.

The most common way of classifying the nanostructures is by dimensionality. Based on dimensionality, the nanostructured materials can be classified into four types. They are zero-dimensional (0-D), one-dimensional (1-D), two-dimensional (2-D), and three-dimensional (3-D) nanostructured materials.

1.1.2.1 Zero-Dimensional (0-D) Nanostructured Materials

These nanostructured materials possess nano dimensions in all the three directions. Examples of these materials are fullerenes, nanoparticles (e.g. gold, silver, nickel, copper, platinum, palladium nanoparticles), metal oxide nanoparticles (e.g. ZnO, TiO₂, Al₂O₃ nanoparticles), quantum dots (a closely packed semiconductor crystal comprised of hundreds of atoms whose size is of the order of a few nanometers, e.g. CdTe, PbSe, CdSe, PbTe), and nanodots (billions of little magnets

which can switch polarity to represent a binary unit of data, e.g. a single digit, 1 or 0). Many of these materials are spherical in shape and have diameter in the range of 1–100 nm. These materials may also have various other shapes such as cubes and polygons.

1.1.2.2 One-Dimensional (1-D) Nanostructured Materials

In these nanostructured materials, one dimension of the nanostructure will be outside the nanometer range and the other two will be in the nanometer range. Examples of these materials are carbon nanotubes, nanowires, nanorods, and nanotubes. These materials are long (several micrometers in length), but their diameter will be few nanometers. Nanowires and nanotubes of metals, oxides, and other materials have been made.

1.1.2.3 Two-Dimensional (2-D) Nanostructured Materials

In this type of nanostructured materials, two dimensions will be outside the nanometer range and the other one will be in the nanometer range. Examples of these materials are graphene, nano films (e.g. coatings and thin-film multilayers), nano sheets, and nano walls. The area of the nano films can be larger (several micrometers squared), but their thickness will be very small (only few nanometers).

1.1.2.4 Three-Dimensional (3-D) Nanostructured Materials

In these nanostructured materials, all the three dimensions will be outside the nanometer range. Examples of these are bulk materials such as nanocomposites, where the sizes of the individual blocks (or structural units) are in the nanometer range, i.e. 1–100 nm.

The summary of classification, description, and examples of nanostructured materials is displayed in Table 1.2.

TABLE 1.2

Classification of Nanostructures Based on Dimensionality

Dimensionality (D)	Description	Examples
Zero dimensional (0-D)	All dimensions in nanometer range	Fullerenes, quantum dots, nanoparticles, nanodots
One dimensional (1-D)	One dimension is not in nanometer range	Carbon nanotubes, nanorods, nanotubes, nanowires
Two dimensional (2-D)	Two dimensions are not in nanometer range	Graphene, thin-film multilayers, coatings
Three dimensional (3-D)	Three dimensions are not in nanometer range	Nanocomposites

1.1.3 SPECIAL NANOMATERIALS

Generally, the special nanomaterials are composed of carbon atoms. Most commonly, they appear in the form of sheets, hollow spheres, or tubes.

The graphene is a two-dimensional nanomaterial made of carbon atoms. It can be considered as a very thin sheet of carbon atoms, arranged in a hexagonal crystal lattice, with a thickness of few nanometers (Figure 1.6).

Fullerenes are spherical, whereas carbon nanotubes are cylindrical carbon nanomaterials (Figure 1.7). Carbon nanotubes can be broadly classified into two types, namely, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). SWCNTs contain one graphene sheet that rolls to form a cylinder. MWCNTs consist of several graphene sheets rolled up together to form concentric cylinders with a large annular gap at center.

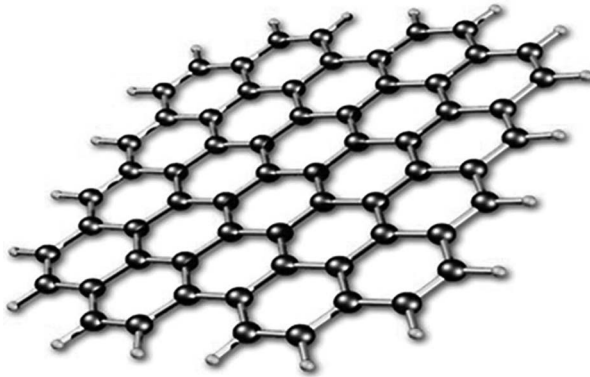


FIGURE 1.6 The layered structure of graphene.

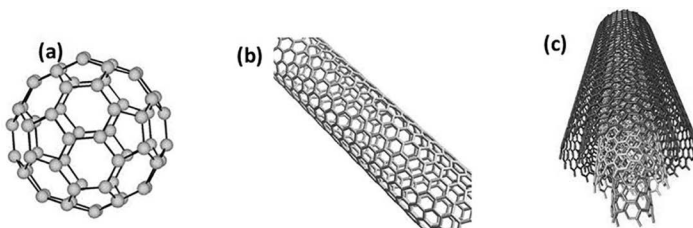


FIGURE 1.7 Structures of (a) fullerene, (b) single-walled carbon nanotube (SWCNT), and (c) multi-walled carbon nanotube (MWCNT).

1.1.4 PROPERTIES OF NANOMATERIALS

The materials such as insulators, semiconductors, and metals have size-dependent physicochemical properties (e.g. electrical, magnetic, mechanical, optical, chemical, and biological properties) below certain critical size. This critical size depends upon the details of the materials, namely, its structure and composition. For most of the materials, this critical size is below 100 nm. At this critical size, the shape of the materials and interactions between nanomaterials decide their properties [3].

When the sizes of the particles are reasonable, say 50 nm and above, their properties will be similar to those of the bulk material. When their size is little smaller, say 10–50 nm, their properties vary linearly with size. However, when the size becomes very small (<10 nm), they display some new and unusual properties due to the quantum confinement effects [4]. Here, the electrons will be confined in a small volume or box, and hence, exhibit energies that depend on the length of box.

The nanomaterials show novel and significantly changed physical, chemical, and biological properties compared to their bulk forms due to their size and structure. For example, (i) gold nanoparticles have much lower melting point compared to bulk gold; (ii) bulk gold is chemically inert, whereas gold nanoparticles are chemically reactive; (iii) metallic bulk gold shines, but gold nanoparticles are non-metallic; (iv) bulk gold appears yellow in colour, but nano-sized gold appears red in colour; (v) bulk iron exhibits magnetic properties, i.e. it is a ferromagnetic material, whereas when the size of particles of iron is reduced to nanoscale, they lose their magnetite properties; and (vii) bulk aluminum is harmless, but when the particles of aluminum are reduced to few nanometers, they can explode.

1.2 BOTTOM-UP AND TOP-DOWN APPROACHES

The properties of a product depend on how its atoms are arranged. For example, if the atoms of coal are re-arranged, then one gets diamond. Similarly, if the atoms in sand are re-arranged with pinch addition of impurities, then one gets computer chips.

Two main approaches used for the synthesis of nanostructures in nanotechnology are bottom-up and top-down approaches [5]. The main difference between conventional technology and nanotechnology is that in conventional technology, top-down approach is preferred, whereas in nanotechnology, bottom-up approach is used. The difference between these two approaches can be explained using an example of powder production, where the crushing and milling of chunks represents an equivalent top-down process, while the chemical synthesis represents bottom-up approach. An illustration of the top-down approach versus the bottom-up approach is provided in Figure 1.8.

1.2.1 BOTTOM-UP APPROACH

The construction of nanomaterials from the bottom, i.e. atom-by-atom, molecule-by-molecule, or cluster-by-cluster, is referred to as bottom-up approach. In bottom-up approach, the atoms or molecules are used as building blocks to produce nanotubes, nanoparticles, nanorods, layered structures, or thin films. Based

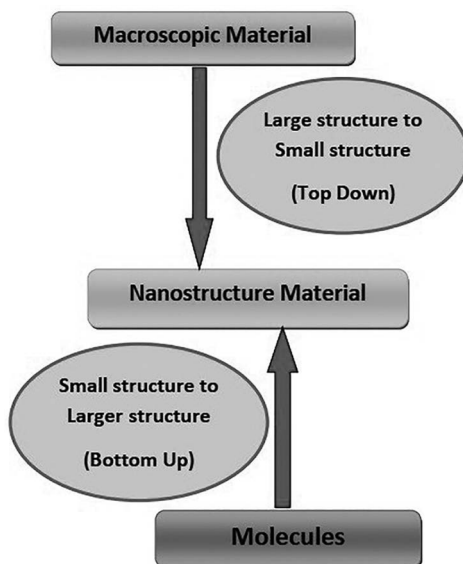


FIGURE 1.8 The top-down approach and the bottom-up approach.

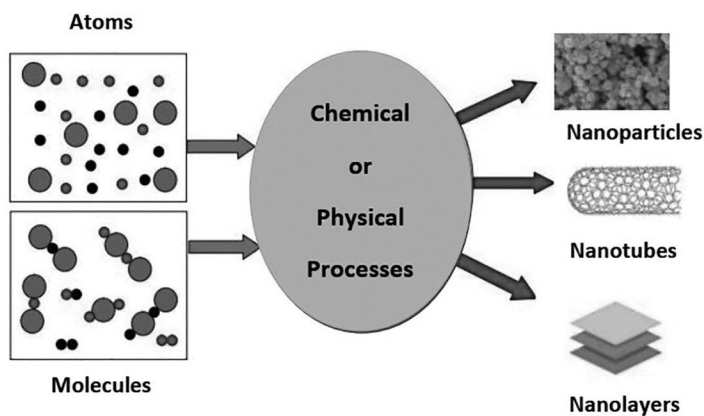


FIGURE 1.9 Bottom-up approach results in particles, nanorods, nanotubes, layers structures, or thin films.

on their dimensionality, these features are also referred to as zero-, one-, or two-dimensional nanostructures. Figure 1.9 demonstrates the building of particles, layers, nanorods, or nanotubes from atoms, molecules, or ions.

Examples for bottom-up approach are as follows:

- i. Sol-gel synthesis, thin-film techniques, spin coating, liquid-phase epitaxy, molecular-beam epitaxy, ion deposition, printing techniques, sputtering techniques, and ion implantation;

- ii. Synthesis of polymer molecule is a typical example for bottom-up approach, in which individual building blocks, i.e. monomers, are assembled to form a large molecule;
- iii. Fabrication of integrated circuits, i.e. assembling different components (e.g. resistors, capacitors, transistors) on a printed circuit board (PCB);
- iv. Crystal growth, where the species either atoms or ions or molecules are orderly arranged to form a desired crystal structure;
- v. Colloidal dispersion in the synthesis of nanoparticles;
- vi. Nanolithography, nano-manipulation, etc.

Though bottom-up approach has tremendous freedom on resulting products, there is limitation on the number of products to be obtained. Bottom-up approaches must be complemented by the self-organization of individual particles in order to obtain ordered structures, where the atoms or molecules arrange themselves to a structure having natural properties.

1.2.2 TOP-DOWN APPROACH

The word “top-down” means starting from bulky pieces of material and producing the required structure by chemical or mechanical methods. The top-down processes have an unmatched flexibility in their application when the structures are within a range of sizes that are accessible by either mechanical tools or photolithography processes. In general, top-down approach is an extension of lithography.

In this approach, size of the bulk piece of materials will be modified to obtain a desired nanostructure. Grinding, cutting, and etching techniques are used for the synthesis of nanostructured materials. Using top-down approach, the nanostructures having sizes in the range of 10–100 nm can be produced.

Examples for bottom-up approach are as follows:

- i. Lithography (using electron beam and ion beam) and ball milling,
- ii. Creating circuits on the surface of a silicon microchip by etching,
- iii. Attrition or milling for making nanoparticles.

1.3 SYNTHESIS OF NANOMATERIALS BY CHEMICAL METHODS

There are physical as well as chemical methods to prepare nanomaterials. However, the most powerful methods are chemical methods. It can be seen from the following that for each type, there are large a number of possibilities. The technique to be used depends on the material of interest, type of nanostructures (0-D, 1-D, or 2-D material), size, quantity, etc.

- a. **Physical methods:** Spray pyrolysis, ball milling, inert gas condensation, flame pyrolysis, arc discharge, thermal evaporation, laser ablation, ion sputtering, pulsed laser deposition, molecular beam epitaxy, etc.

- b. **Chemical methods:** Solvothermal synthesis, photochemical synthesis, sonochemical synthesis, chemical vapour deposition (CVD), sol-gel process, etc.

1.3.1 SOLVOTHERMAL SYNTHESIS

Solvothermal synthesis is a versatile method for controlling the morphology of the well-defined nanostructure materials, e.g. metal oxide and metal nanoparticles. In this synthesis, the chemical reaction is usually performed in a sealed container such as an autoclave or a bomb, where the temperature of solvent is well above its boiling point [6] and at pressure above 1 bar. If water is used as solvent, then it is called as hydrothermal process. In this synthesis, the reactivity and solubility of the reagents can be increased with increase in temperature and pressure, which may lead to unexpected reactions compared to classical routes [7].

1.3.2 PHOTOCHEMICAL SYNTHESIS

The photochemical synthesis depends on the absorption of photons belonging to ultraviolet spectral region by a photochemically active material which can undergo either decomposition or re-arrangement into reactive species, namely, radicals. These radicals can initiate the chemical reactions leading to the formation of various intermediate products [8]. The main advantages of photochemical synthesis include (i) performing the chemical reactions without controlling temperature precisely, (ii) easy to up-scale, and (iii) control over the synthesis by changing the intensity of radiation.

1.3.3 SONOCHEMICAL SYNTHESIS

The word “sonochemical” in the name indicates chemical reaction taking place by the use of sound waves. In this method, the synthesis of nanomaterials will be initiated by ultrasonication in liquids utilizing both the chemical and physical effects of ultra sound arising from the impulsive collapse of bubbles produced by acoustic cavitation [9]. The extremely high temperature and pressure generated inside bubbles offer distinctive conditions for the synthesis compared to other chemical processes.

1.3.4 CHEMICAL VAPOUR DEPOSITION (CVD)

CVD can be utilized to obtain coatings of various materials, namely, organic or inorganic materials on some substrates [3]. It is popularly used in industry due to its economical viability, ease of processing, simple instrumentation, and possibility of depositing different types of materials. The single crystalline films or nanocrystalline films can also be grown by this process by maintaining suitable conditions.

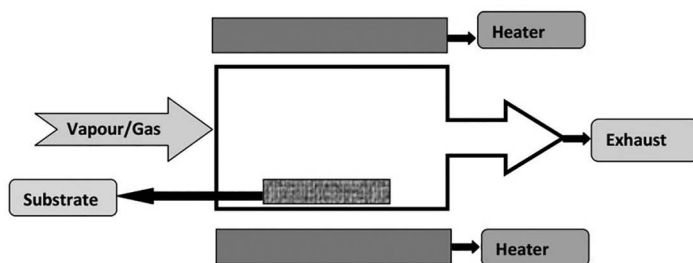


FIGURE 1.10 A schematic of chemical vapour deposition (CVD) process.

In CVD process, reactant gas or vapour will be made to transport towards the substrate which is kept at a high temperature where the reactant gas or vapors crack into various products and diffuse on the surface of the substrate, and nucleate and grow to form required film of material. The various by-products created during the process will be removed through the exhaust.

In this process, gas or vapour of the preferred material will be pumped into the reaction chamber using a carrier gas (Figure 1.10). In several cases, the reaction may occur through aerosol formation in gas phase. The CVD proceeds by different processes such as reduction of gas, chemical reaction between various source gases, oxidation, etc. However, the reaction at the substrate is preferred compared to that in the gas phase. Generally, temperatures and gas pressures in the ranges of 400°C–1200°C (at the substrate) and 100 m Torr to 1 Torr are used. The substrate temperature and gas pressure are the critical factors for the growth rate and film quality. The growth rate is limited by kinetics of surface reaction at low temperature and mass transport, i.e. supply of reactant gas or vapor to the substrate, at intermediate temperature. Desorption of precursors from the substrate surface will reduce the growth rate at high temperatures.

Based on the types of precursors used, the applied deposition conditions, and the forms of energy supplied to the system to activate the chemical reactions, there are several derivative methods of CVD. They are as follows:

- i. **Metal organic chemical vapor deposition (MOCVD):** when metal organic compounds are used as precursors
- ii. **Plasma enhanced chemical vapor deposition (PECVD):** when plasma is used to promote chemical reactions
- iii. Low pressure chemical vapour deposition (LPCVD)
- iv. Aerosol-assisted chemical vapour deposition (AACVD)
- v. Laser assisted chemical vapour deposition (LACVD)
- vi. Catalytic chemical vapour deposition (CCVD).

1.3.5 SOL–GEL PROCESS

The sol–gel process involves two types of materials: *sol* and *gel*. Sols are solid particles present in a liquid and are the subclass of colloids. Gels are a continuous network of particles having pores filled with liquid, for example, polymer

containing liquid. In sol-gel process, the sols in liquid are formed first, and then, sol particles are connected with each other to form a network [3]. Powers, thin films, or monolithic solids can be obtained after drying the resultant material.

The sol-gel method is useful for the preparation of ceramics, glass materials, and metal oxides. This method is used for the fabrication of different nanostructures, where metal alkoxides and metal chlorides are generally used as precursors.

1.4 PHYSICOCHEMICAL PROPERTIES OF NANOPARTICLES WITH REFERENCE TO BIOLOGICAL SYSTEMS

The behaviour of nanoparticles changes in *in vivo* environment. The morphological parameters like shape, size, and texture influence the nanoparticle behavior in biological systems [10]. The properties of nanoparticles are the reason for the degradation and movement kinetics in biological systems [11]. The cell signaling is dependent on the shape and size of nanomaterials [12]. The presence of ligands is influenced by the surface properties of nanoparticles in biological systems [12,13].

1.4.1 SIZE AND DISPERSION ABILITY

Nanoparticles are supposed to be in normal flow till they reach targets. Size plays a major role in the nanoparticle elimination. The studies have shown that the particles having size greater than 7 μm are separated mechanically by the capillary network of the internal organs, whereas particle size smaller than 100 nm remains in blood vessels [14,15]. The size of nanoparticles affects their speed, diffusion, and adhesion [16]. The nanoparticles smaller than 6 nm are restricted by glomerular filtration in the kidney [17]. There is a confusion regarding the internalization of gold nanoparticles with respect to size, and there is no correlation exists between size and action [17]. The asymmetry might be explained by using various cell types with biological characteristics. The surface area and nanoparticle diameters play a crucial role in the targeted drug delivery. The degradation of a nanoparticle matrix is responsible for the release of a drug.

The interaction of materials with biological systems solely depends on their shape and size. The reduced size of nanomaterials results in an increase in the surface area to volume ratio, which makes the surface self-reactive. The particle size and surface area of nanomaterials decide how a biological system responds [18]. It has been reported that different biological mechanisms are dependent on the size of nanomaterials [19].

1.4.2 SHAPES

There are major challenges in understanding the correlation between shape and size for efficient development of nanoparticle-based delivery systems of drugs. The shapes of nanoparticles affect delivery mechanisms and degree of toxicity in biological systems [20–22]. The studies on toxicity related to shape have been reported for various nanostructures like nanotubes, silica, allotropies, nickel, gold, and titanium [17,23–25].

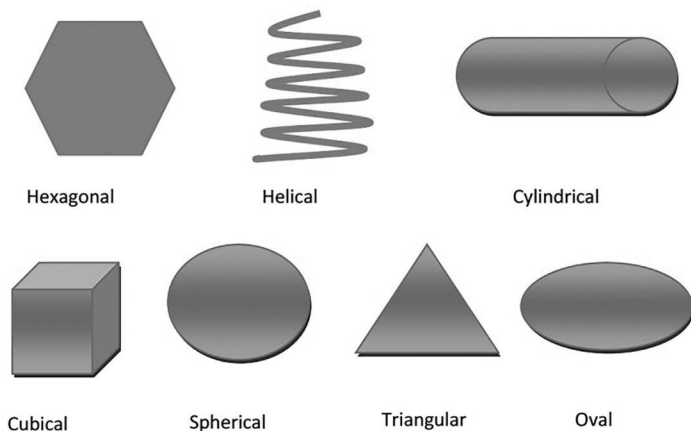


FIGURE 1.11 The various shapes of nanoparticles.

Many reports suggested a significant role of various shapes, namely, oval, cubical, triangular, helical, spherical, hexagonal, cylindrical, and circular (Figure 1.11), in the circulation and flow of nanoparticles in biological systems [26–28]. The influence of the shape of nanoparticle flow mechanism has been studied by various research groups [14,29,30].

The various investigations suggest that spherical particles move easily in biological systems, whereas particles with irregular shapes and geometries have the possibility to align, tumble, and follow irregular paths in biological systems [29]. The minimum diameter of a spherical nanoparticle must be less than 200 nm to pass through the spleen [30]. For a nanoparticle having disk shape, if the diameter is 7 μm and the height is 152 μm , then it can pass through the organ [31]. It is also evident that nano-spherical and nano-cylindrical particles are internalized easily than longer particles *in vitro*. The circulation time of nanoparticle-based injections solely depends on the shapes of the nanoparticles (Figure 1.12). The unfolding effects created by cubic nanoparticles were higher than those by their spherical counterparts [31]. Recent research reveals that nanoparticles with different shapes but the same chemical compositions show different cytotoxic behaviors. Nanotubes and nanowires show higher toxic behavior than spherical ones [32]. The studies have been conducted to understand the effect of non-spherical nanoparticles in the delivery of antitumor drugs [31].

1.4.3 SURFACE CHARGES

The toxicity of nanoparticles solely depends on the surface charge and defines the interaction with biological systems. The different aspects of nanomaterials like selective adsorption [33,34], plasma-protein binding [34], colloidal behavior, integrity of blood–brain barrier, and the permeability of membrane are controlled by the surface charge of nanoparticles [35]. The cellular uptake of positively

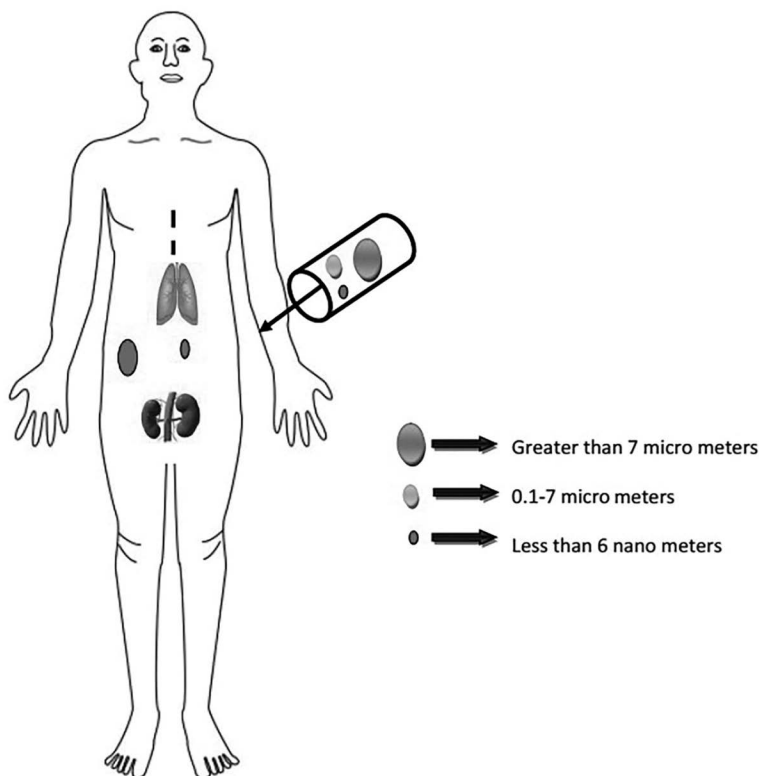


FIGURE 1.12 Administration of various particle sizes in human body.

charged nanoparticles is significantly higher than those of negatively charged and neutral particles. The surface charge and hydrophobicity are taken as references to characterize nanoparticles [36,37]. Hydrophobicity of nanoparticles has huge relevance and advantage to get excreted from biological systems. The reduction of interaction of biological systems with protein is due to the decrease in hydrophobicity [38]. The electrical potential at the surface of nanoparticle, called zeta potential, creates tendency for aggregation of particles [39]. The repulsive force between nanoparticles increases with increase in the electrical potential of surfaces [39]. The internalization of positively charged nanoparticles is much more effective than negatively charged or neutral nanoparticles [40,41]. Apart from charge, the internalization also depends on the cell [36,42].

1.4.4 TARGETING MECHANISM

Nanoparticles have the ability to be targeted to specific sites in biological systems and reduce the side effects on healthy tissues. The targeting approaches may be passive or active. The structural and physicochemical characteristics of the target

sites are exploited in the case of passive targeting [43]. For effective targeting, the reduced size of nanoparticles takes the advantage of permeation and retention mechanism [44]. The linking molecules such as ligands, antibodies, peptides, engineered particles, nutrients, carbohydrates, proteins, or aptamers to the surface of nanoparticles is observed to be better carriers in active targeting [41,43]. The advantages of the specific interaction of molecules with the surface of the nanoparticles are exploited in active targeting [45].

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2 Innovations in Nanotechnology for Biomedical Sensing, Imaging, Drug Delivery, and Therapy

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2.1 INTRODUCTION

Nanotechnology takes advantage of the unique processes and characteristics including physical, chemical, and/or biological properties of nanometer-scale materials to create custom structures, instruments, and programs. Nanomaterials can vary from 1 to 100 nm in size. The manipulation of materials at this small scale enables accurate production of compounds. Scientists in the biological sciences are implementing advances in nanotechnology both in the laboratory and in medicine. Several of these advances and implementations have been under development (Whitman et al. 2008).

Nanotechnology has a significant effect on the development of a new generation of biosensors classified as nano-biosensors. Nano-biosensors typically contain a molecule of biological detection immobilized on the surface of a signal transducer. The interaction between the biorecognition agent and the analyte is a diverse process, and thus, the nature of the biosensing interface is important for determining the efficiency of the nano-biosensor. Nano-biosensors are commonly used for the molecular identification of biomarkers correlated with disease diagnosis. The introduction of modern nanomaterials to biosensing has influenced biosensing science. The use of high-surface nanomaterials was significant in the development of nano-biosensors with higher sensitivity and quicker turnaround times (Prasad 2014).

A promising area of use of nanotechnology is currently being applied to innovative methods of medical imaging. Nanomaterials may be significant components to medical imaging due to their unique qualities of biocompatible size distribution, high penetration power, image contrast efficiency, surface tuning, stability, and half-life. Such technologies allow nano-based imaging to track live physiological processes in a non-invasive way, with almost no discomfort, which is not only essential for proper diagnosis or treatment, but also useful for basic understanding of clinical disorders, which, in effect, may help to develop sophisticated methods in the future (Deb, Ghosh, and Shetty 2015).

In nanomedicine, nanomaterials are used for controlled drug delivery. This type of drug delivery and therapy has made successful adjustments in clinical settings through the implementation of novel methods for diagnosis and treatment, enabling unmet medical requirements to be addressed, incorporating active molecules that could not be used due to their high toxicity, using various mechanisms

of action, optimizing effectiveness/bioavailability, and minimizing dose and toxicity (Soares et al. 2018).

The early influence of nanotechnology on medicine is starting to be observed, with new nanoscale therapeutic and diagnostic approaches currently under development or in clinical practice. This chapter examines the field of nanotechnology for biomedical sensing, imaging, drug delivery, and therapy from the perspective of what these technologies are, how they are used in biomedical sciences, and discoveries and advancements made.

2.2 NANO-BIOSENSING

2.2.1 NANO-BIOSENSORS

Biosensors are developed to identify or measure biological entities such as DNA sequences, enzymes, and proteins. Biosensors are instruments or techniques used to analyze biological responses and convert them into electrical signals. These sensors need to be highly specific, where external factors such as pH and temperature should not affect the results (Mehrotra 2016). The operating theory is to attach bioanalytes of concern to bioreceptors, which in effect modulate the physiochemical signal correlated with the attachment. The transducer represents and converts the physiochemical signal into an electrical signal. Signal differences like electrical potential, current, conductivity, resistance, electromagnetic radiation intensity and duration, temperature, and density are controlled. The shifts in one or more of these parameters are used to measure the presence or absence of bioagents (Prasad 2014).

Current advancements in nanotechnology and advanced electronics manufacturing technologies converge with the creation of a new range of biosensors named nano-biosensors and a new era in bio nanotechnology for disease diagnosis. Biochemical or biological processes can be measured by nano-biosensors integrated into a lightweight detector using any mechanical, optical, or magnetic system. Nanostructures in nano-biosensors serve as an indirect framework among biological agents and physicochemical detectors (Kumar 2010, Prasad 2014). The use of diverse nanomaterials for biosensors has enabled faster detection and its reproducibility due to the unique properties such as high electrical conductivity, better shock bearing ability, and sensitive responses (Malik et al. 2013).

2.2.2 NANO-BIOSENSORS USED FOR BIOMEDICAL APPLICATIONS

In a nano-biosensor (Figure 2.1), the signaling system that refers to the material being tested is of biological origin. To capture a clearly recognizable signal, it must be attached to a transducer. Nanomaterials are used as the transducers. Nano-biosensing agents currently used in the biomedical field include nanoparticles, nanowires, graphene and carbon nanotubes, and nanochannels.

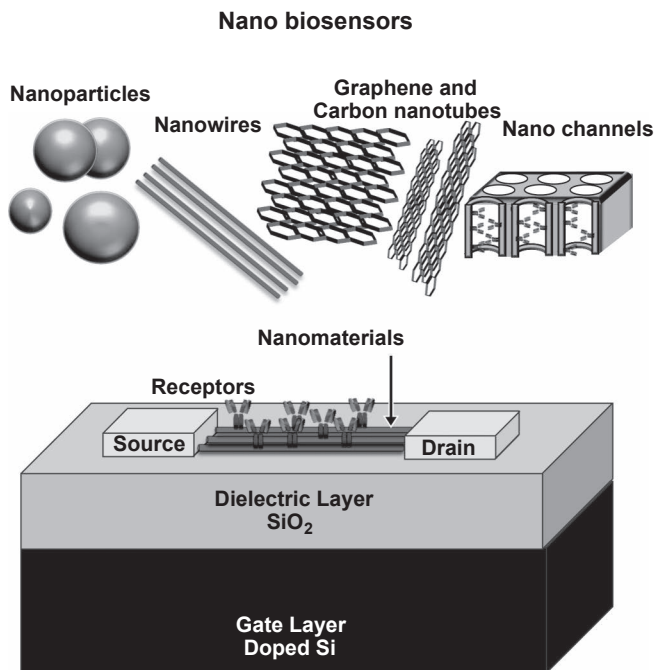


FIGURE 2.1 Nanomaterials such as nanoparticles, nanowires, graphene and carbon nanotubes, and nanochannels serve as transducers in a nano-biosensing device. The nanomaterial reflects and transforms the physiochemical signal of the bioanalyte into an electrical signal where the signal change shows the absence or presence of the biological object.

2.2.2.1 Nanoparticles

Nanoparticles are deemed clusters of atoms or molecules between 1 and 100 nm in scale. These particles can be composed of various agents including metals, carbons, and polymers (Chamorro-Garcia and Merkoçi 2016). Nanoparticles received attention for applications of biosensing due to their intriguing properties. Such properties include a large surface area that improves biorecognition and receptor immobilization, strong catalysis and electron transfer performance, and good biocompatibility. Using nanoparticles as a biosensing agent may lead to major signal amplification, increased sensitivity, and significant increases in the identification and quantification of biomolecules and various ions (Malekzad et al. 2017). The most popular nanoparticles used for biosensing are gold nanoparticles, which have been used in the development of optical and electrochemical biosensors. The overall effect that was seen using gold nanoparticles was the increase in detection signal, or response, for analytes that are especially low in concentration (Su et al. 2017). Fluorescent signals were particularly enhanced when using Quantum Dots. These are colloidal semiconductor nanocrystals

that present fluorescent properties applicable to biosensing. These properties are achieved due to the Quantum Dots having an inorganic core that is surrounded by an outer surfactant layer which stabilizes the molecule and acts as a ligand for the second coating of the semiconductor shell (Reiss, Protiere, and Li 2009). Quantum Dots can then be functionalized with biomolecules directed toward the bioagent of interest (Blanco-Canosa et al. 2014).

2.2.2.2 Nanowires

Nanowires are made from a variety of conducting and semiconducting materials, where their surface can be readily modified to become sensitive to chemical and biological species. Nanowires offer the highest chance of producing strong, responsive, and accurate electrical detection of biological binding events due to their unique properties. These properties include having the same thickness as biological macromolecules, tunable conductive properties, and the capacity to attach analytes on their surface providing a simple, label-free electrical measurement (Cui and Lieber 2001). Metallic-based materials are used to make the source and drain, isolated by a thin silicone layer attached to a receptor that can sense specific charged species such as DNA and RNA. The binding of a charged species to the receptor induces an electric field to the wires altering their conductivity (Ambhorkar et al. 2018). This current flowing through the conductor is extremely sensitive to small disruptions, and in nanowires, current flows are particularly close to the surface, resulting in increased sensitivity at low ion power (Gooding 2006, Ramanathan et al. 2005).

2.2.2.3 Graphene and Carbon Nanotubes

Graphene and carbon nanotubes consist of carbon atoms configured in hexagonal networks of atomic-size planar structures, where graphene's structure represents a sheet and carbon nanotubes are rolled-up graphene sheets (Georgakilas et al. 2015, Novoselov et al. 2012). Their structure allows for having a high surface-to-volume ratio, innovative electronic and electron transport properties, pliability, impermeability, mechanical strength, and excellent thermal and electrical conductivities (Chen, Feng, and Li 2012, Li et al. 2015). Carbon nanomaterials can be used by increasing the roughness and active area of a sensor surface allowing for enhanced electrocatalytic and enzymatic properties (Ambrosi et al. 2014), where graphene flakes or Quantum Dots, due to their quenching properties and small size presenting with quantum confinement, are used in fluorescent-based biosensing (Georgakilas et al. 2015, Morales-Narváez and Merkoçi 2012). These carbon nanostructures are strongly influenced by small surface disturbances, such as those associated with the mixture of macromolecules. This offers rapid (real-time) and sensitive label-free bio-electronic recognition and strong stability in nano-sensor arrays (Desai, Hansford, and Ferrari 1999).

2.2.2.4 Nanochannels

Nanochannels or protein-based ion channels are used in living systems for electrical signaling. Channels with diameters within 1 and 100 nm are considered

nanopores, if the pore diameter is larger than the depth. These channels have inspired integration of natural ion channels and artificial electrical biosensing systems based on both single nanochannel and nanochannel arrays. In most cases, polymeric membranes are used as substrate materials to generate nanochannels (de la Escosura-Muñiz and Merkoçi 2012, 2016). The sensing mechanism applied is based on the recording of the change in electrical conductance when a microscopic particle passes through the channel. The change in electric pulse is related to the nature and properties of the particle (Kasianowicz et al. 2008). The most promising biosensing systems using nanochannels are single nanochannel-based electrochemical systems where advantages are seen in sensitivity, real-time sensing, and multiple-analyte analysis (de la Escosura-Muñiz and Merkoçi 2012).

2.2.3 RECENT APPLICATIONS AND DISCOVERIES OF NANO-BIOSENSORS IN BIOMEDICAL SCIENCE

Over the next decade, nanotechnology-based analytical instruments will become usable and will be capable of performing thousands of tests very quickly and affordably. Future trends in diagnostics will proceed in the miniaturization of nanoscale biochip technologies. Molecular electronics and nanoscale chemical sensors would enable the development of microscopic sensors capable of detecting chemical patterns in the fluid. Data from many other instruments streaming passively into the bloodstream make it possible to quantify the properties of tiny chemical inputs in the amount of macroscopic tissue. Estimates of realistic system capabilities have been used to determine the efficiency of standard chemicals released into the blood by tissues in response to localized damage or infection. Such results show that devices can reliably distinguish a single cell-sized chemical source from the background chemical concentration *in vivo*, offering high-resolution sensing in both time and space (Jain 2013b). This is also true for fluorescent labeling as miniaturization reduces signal intensity, but some improvements have been made to make fluorescent labeling methods viable with nanomaterials. Nano-biosensors will also facilitate the development of diagnostic non-polymerase chain reaction technologies. Additionally, nanotechnology can theoretically be used for single-cell analysis to enable genetic diagnosis (Prasad 2014).

Cancer diagnosis will be an important area of nano-biosensing. Tools for molecular cancer diagnosis, including genetic testing, are widely commercially available. Nevertheless, when a cancer is diagnosed using current available tools, it is often too late for curative therapy. Nano-biosensors are ultra-sensitive in the identification of cancer biomarkers and so may be used for early detection and cancer treatment in the future. Nano-systems are now at the feasibility level for this reason. A nano-device for use in both cancer diagnostics and therapeutics, known as a nano-theranostic technology, could be inserted as a prophylactic intervention in patients who do not have any signs of cancer, and cancer screening could be done through remote monitoring. These tests could allow for cancer detection at the earliest stage, as well as allow for effective therapeutic

intervention. Such monitoring devices should be biodegradable, and protection should be developed prior to implantation. Such a surveillance system would be the pinnacle in customized cancer prevention. Early detection would increase the chances of a cure (Jain 2013a). Biosensors based on nanotechnology would not only improve diagnosis but also help to link diagnosis with treatment and the development of personalized medicine.

2.3 NANOTECHNOLOGY IN MEDICAL IMAGING

2.3.1 NANOTECHNOLOGY AND BIOMEDICAL IMAGING

One of the main current work focuses of medical diagnostics is molecular imaging, where molecular imaging can promote early diagnosis, classify the stage of illness, offer essential information on clinical processes, and can be used to track the effectiveness of treatment. Molecular imaging relies heavily on the creation of sophisticated probes required to identify cellular and molecular biological processes (Cormode et al. 2009). The growing need for disease identification and classification tends to drive the production of imaging and contrasting technologies. The manufacturing of nontoxic nanomaterial contrast agents with a longer circulation period can overcome obstacles and allow for quick and accurate imaging of tissue microstructures and lesion classification (Han et al. 2019).

2.3.2 NANOMATERIALS USED IN MOLECULAR IMAGING

Various synthetic and natural nanomaterials are being implemented to enhance molecular imaging (Table 2.1) through increased attenuation, tissue targeting, biocompatibility, and surface functionality.

2.3.2.1 Gold Nanoparticles

Gold nanoparticles recently have gained significant attention in the use of various imaging techniques thanks to their surface composition, biocompatibility, incremental adverse effects, positive charge, and strong attenuation coefficient (Ahmed et al. 2018). X-ray computer tomography (CT) is used to interpret variations in tissue density, which provide an X-ray attenuation differentiation between soft tissue and electron-dense bone. It is necessary to improve the contrast between diseased and healthy tissues. Organic iodine molecules are commonly used as CT contrast modulators but are short-lived due to rapid renal clearance and non-specific vascular permeation. Researchers who investigated gold nanoparticles found that these particles had blood clearance through renal excretion without concentration in other vital organs, increased stability, and that these pegylated particles had increased blood circulation times. Furthermore, gold nanorods coated with an antibody showed increased attenuation coefficient compared to non-targeted gold nanorods (Nune et al. 2009). Enhanced X-ray attenuation in target cells compared to normal cells supports the basic premise to produce molecular X-ray CT imaging agents.

TABLE 2.1
Nanomaterials Implemented in Various Molecular Imaging Applications
Providing Enhanced Imaging Effects

Nanomaterial	Imaging Application	Effect	References
Gold nanoparticles	X-ray computer tomography	Strong attenuation coefficient and improved contrast	Ahmed et al. (2018), Nune et al. (2009)
Quantum Dots	Fluorescent imaging	Good fluorescence quantum yield	Resch-Genger et al. (2008)
Iron oxide nanoparticles	MRI contrast agent	Effective contrast agent with tunable and high magnetization	Stephen, Kievit, and Zhang (2011), Wahajuddin and Arora (2012)
Carbon nanotubes	Field emission/ electron microscopy Fluorescent imaging	Fast electrical and thermal transmittance, NIR fluorescence properties	Abdalla et al. (2015), Gong, Peng, and Liu (2013)
Dendrimers	MRI contrast agent	Increased half-life and magnetic intensity	Nune et al. (2009)

2.3.2.2 Quantum Dots

Quantum Dots are semiconductor crystals of nanometer dimensions with distinctive conductive properties determined by its size. They have near-unit quantum yields and are 10–100 times brighter than most dyes. Quantum Dots also exhibit wide absorption properties, short line width in emission spectra, continuous and tunable emission maxima due to quantum-scale effects, relatively long fluorescence life, and marginal photo-bleaching (Resch-Genger et al. 2008). Such characteristics make Quantum Dots useful in medical imaging systems once combined with the ability to target specific cells *in vivo* and conjugation with associated bioactive groups. There are chemical optimization techniques for the precise control of Quantum Dot size and composition, which ultimately affect the absorption and emission properties. This may enable researchers to use different Quantum Dot properties for applications such as cell marking, biosensing, and nucleic acid detection (Zhu et al. 2013).

2.3.2.3 Iron Oxide Nanoparticles

Magnetic nanoparticles gained support for their future use in optical, magnetic, and electronic equipment due to their biocompatibility at low concentrations, strong magnetic density, and surface functionality. They can be functionalized with antibodies, nucleosides, proteins, and enzymes for the diagnosis of diseased tissues such as cancer (Markides, Rotherham, and El Haj 2012). Superparamagnetic iron oxide nanoparticles (SPIONs) have broad magnetic moments and are useful as contrast agents in MRI, which is used for measuring tissue structure and provides

better contrast between soft tissues compared to X-ray CT. *In vivo*, SPIONs may be directed to the reticuloendothelial system (RES) as defined by the size of the particle and its surface chemistry (Stephen, Kievit, and Zhang 2011). SPIONs have a high magnetization concentration and magnetization loss in the absence of a magnetic field, and these nanoparticles are thought to be comparatively less harmful than optical imaging agents. Ultra-small iron oxide nanoparticles (USPIONs) have been used to overcome non-specific uptake, clearance, and enhance tumor extravagance. Engineered nanoparticles with high and tunable magnetism give increased resilience, are biologically nontoxic, and have lower dosage requirements when compared to conventional iron oxide contrast agents (Wahajuddin and Arora 2012).

2.3.2.4 Carbon Nanotubes

Carbon nanotubes possess special physical, electrical, and mechanical properties. The tubes consist of single graphite layers rolled into a tube, where the structure can either be single-walled or multi-walled (Saifuddin, Raziah, and Junizah 2012). Exceptional properties of carbon nanotubes, including fast electrical and thermal transmittance and high strength under tension, suggest the possibility for their use as contrast agents in field emission/electron microscopy, scanning probe microscopy tips, nano-transistors, and composite material (Abdalla et al. 2015). Recent developments in biomedical imaging have centered on the near infra-red (NIR) fluorescence properties of single-walled carbon nanotubes (SWNTs) and surface functionalization. NIR fluorescence is contained in a bio transparent area (700–1300 nm) where autofluorescence, absorption, and dispersion of blood and tissue are reduced. The unusual NIR fluorescence properties of SWNTs make them attractive as contrast imaging and biological sensors (Gong, Peng, and Liu 2013). Surface functionalized multi-walled carbon nanotubes (MWNTs) have been successfully used for bio-imagery purposes and overcame solvent insolubility forming supramolecular complexes with biological macromolecules and substrates based on electrostatic interactions with no cytotoxicity (Vardharajula et al. 2012). Bio-imagery technologies utilizing carbon nanotubes have been established for the degradation, identification, and dynamic imaging of cancer cells using SWNTs that are covalently bound to visible-wavelength fluorophores (Kam et al. 2004).

2.3.2.5 Dendrimers

Dendrimers are well-established polymers with compact and symmetrical molecular conformations that are synthesized with precise structural control and monodispersity. Adjustable variability in size, existence of interstitial spaces, flexible composition, cell internalization, selectivity towards cells and intracellular components, protection of compounds, and controllable release of materials make dendrimers attractive particulate structures for biomedical applications (Janaszewska et al. 2019). Through taking advantage of the natural, uniform nature of dendrimers, several of the limitations related to low-atomic-weight contrast agents and imprecise synthetic polymers can be resolved. Evidence has

shown that the use of dendrimer-based contrast agents has shown longer half-lives than conventional agents. The reliability of dendrimer-based contrast agents was assessed by the size that affected the pharmacokinetics, permeability, clearance, and RES uptake. Targeted dendrimers have also been studied for biomedical imaging. Reports have shown an overall increase in magnetic intensity specific to the target site (Nune et al. 2009).

2.3.3 RECENT ADVANCEMENTS AND DIRECTIVES OF BIOMEDICAL APPLICATIONS OF NANOMATERIALS IN MOLECULAR IMAGING

Over the past decade, advancements in nanotechnology in biomedical imaging have been greatly enhanced and provided precise molecular imaging with significantly increased contrast improvements in conventional imaging agents. Numerous reports have found tumors in animals at earlier stages relative to the medical methods used today (Han et al. 2019). While the industry progresses, advancements in contrast agent developments, associated with molecular targeting, will continue. While recent developments in biomedical imaging using nanomaterials seem encouraging, more attention will need to be paid to techniques and designs for measuring and manipulating pharmacokinetics, biodistribution, and toxicity. Initial measures have been taken to formulate the classification of nanomaterials used in bio-imagery, and validated approaches provide an excellent benchmark for the systematic study of nanomaterials for biological purposes (Nune et al. 2009).

The creation and development of closely designed nanomaterial-based contrast agents for biomedical imaging requires a broad range of skills from science and biophysics to technology and radiology. Manifestation of this interdisciplinary study design will be needed to advance the products used in this type of imagery. The design strategy to the development of biomedical imaging agents may involve the construction of new medicinal drugs; however, more problems will occur in resolving colloid stability and specificity of conjugated ligands. As nanomaterial contrast agents move to the market, difficulties with the reproducible large-scale production of these products, which retain a high value-to-cost ratio, may emerge. In fact, scientists will need to be cautious of possible issues such as occlusions, renal toxicity, and hepatotoxicity, which may not be observed in early animal experiments (Patra et al. 2018).

In order to increase the chance of nanomaterials to clinical translation, their concentrations should be minimized to improve imaging performance by improving the contrast-to-noise ratio, steering the agent to tissues of concern, or enhancing the responsiveness of the device. Researchers should also acknowledge the possible risks involved with the implementation of advanced imaging techniques and recognize the challenge of processing and interpreting large data sets collected using nanomaterial contrast agents (Rosen et al. 2011). As with any drug for human consumption, comprehensive health and toxicology tests would include

concurrent pharmacokinetic and biodistribution research in early clinical trials. Ultimately, new methods employing multifunctional nanomaterials (e.g., visualization and therapeutic) will take full account of the operational nature of these forms of hybrid strategies.

2.4 NANOMATERIALS FOR DRUG DELIVERY AND THERAPY

2.4.1 NANO-DRUG DELIVERY AND THERAPY

Nanomedicine is described in a number of different ways, but a comprehensive description contains the advancement of nanomaterials, nanostructured surfaces, and nanoanalytical strategies for biochemical diagnosis, treatment, follow-up, and medication (Riehemann et al. 2009). Nanoscale molecules used in nanomedicine have revolutionized drug delivery, enabling pharmacological agents to be preferentially aimed at organ-, tissue-, and cell-specific levels, while minimizing the exposure of healthy tissues to medicines (Anderson, Burdick, and Langer 2004). The use of nanomaterials for the delivery of drugs is intended to overcome limitations of bio-macromolecular therapy, including short plasma half-life, poor stability, and probable immunogenicity. While traditional drug delivery mechanisms are efficient in the controlled release of drugs to generate high local concentrations, their complexity is restricted to targeting tissues instead of single cells (Goldberg, Langer, and Jia 2007).

2.4.2 NANOMATERIALS IN BIOMEDICINE DELIVERY AND THERAPY

There have recently been major innovations in the area of delivery systems to provide pharmacological agents or biologically active compounds for their target locations for the treatment of different diseases (Obeid et al. 2017). Several drug delivery systems have been effectively used, but there are still some obstacles that need to be addressed and newer technologies need to be explored for the safe delivery of drugs to their specified sites. As a result, nano-based therapeutic systems are essentially being researched to promote advanced drug delivery systems (Patra et al. 2018). Nanomaterial architecture has been thoroughly researched and is by far the most advanced technology in the field of nanomaterial applications due to its practical benefits like the capability to change characteristics, such as solubility, drug delivery features, diffusivity, pharmacokinetics, and immunogenicity. As a consequence, this can lead to progress and production of easier routes of delivery, reduced risk, fewer side effects, better biodistribution, and an extended life-cycle of medications (Mirza and Siddiqui 2014). There are several biopolymeric and nanomaterials that are utilized in the drug delivery systems. These include but are not limited to protein and polysaccharide nanoparticles (Chitosan, Alginate, Xanthan gum and Cellulose), liposomes, dendrimers, inorganic/metallic nanoparticles, nanocrystals, and carbon nanotubes (Figure 2.2).

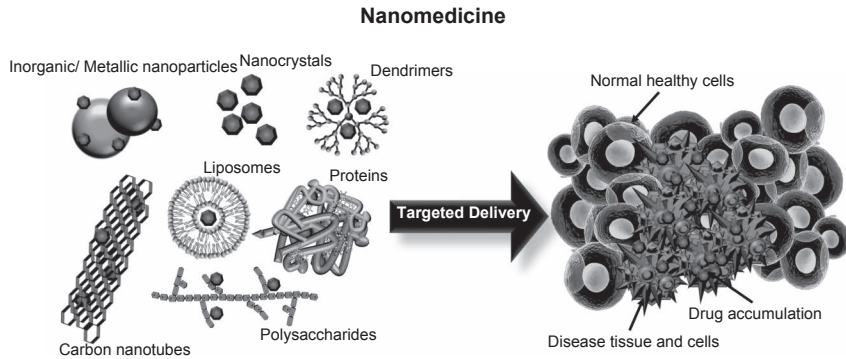


FIGURE 2.2 Nanoparticles used for nanomedicine include protein and polysaccharide nanoparticles, liposomes, dendrimers, inorganic/metallic nanoparticles, nanocrystals, and carbon nanotubes. Nanomaterial-based drug delivery systems are used to encapsulate therapeutic drugs for site-specific targeted distribution of medications and improved bioavailability.

2.4.2.1 Protein and Polysaccharide Nanoparticles

Polysaccharides and proteins are commonly referred to as organic polymers and are therefore derived from organic materials such as plants, insects, microorganisms, and aquatic resources (Bassas-Galia et al. 2016). Protein-based nanoparticles are typically degradable, can be metabolized, and easy to manage due to their ability to bind specific drugs and other target receptors (Lohcharoenkal et al. 2014). In the same way, polysaccharides are comprised of sugar bases connected by O-glycosidic bonds. Both the structure and biological sources of such monomers are capable of conferring on these polysaccharides a series of specific physicochemical properties (Liu et al. 2008). Protein nanoparticles can be synthesized into biodegradable polymeric microspheres made from various proteins such as bovine serum albumin, making them water soluble and gliadin or zein making them water insoluble depending on the application, providing controlled and sustained drug release (Verma et al. 2018). One of the biggest drawbacks when using polysaccharides in nanomedicine is its high-temperature deterioration properties, which are often needed within industrial applications. In fact, most polysaccharides are water-soluble, which limits their use in some areas of nanomedicine, such as tissue engineering. Nevertheless, methods such as cross-linking of polymer chains have been used to improve the integrity of polysaccharide chains as well as maintain their consistency in aqueous conditions (Pertici 2017, Azeredo and Waldron 2016). Below are some of the polysaccharides that are applied in drug delivery and therapy.

2.4.2.1.1 Chitosan

Chitosan is a natural biopolymer that can be easily adapted to reach a desired target. Chitosan has a positive surface charge provided by key amino groups in a

polymer backbone. It has a complex structure, a polycationic base, and the ability to form inter- and intramolecular H-bonding (Jiang et al. 1996). New methods to produce chitosan nanoparticles involve ion crosslinking, covalent crosslinking, reverse micellar phase, precipitation, and emulsion-droplet coalescence. Chitosan nanoparticles have been shown to have a variety of uses in the treatment of drug delivery and other biological functions. Chitosan nanoparticles that are biodegradable, biocompatible, less harmful, simple to produce, and so are possible efficient drug delivery methods. The functional value of the active ingredient may be improved by increasing its stability, solubility, and bioavailability, alongside site-specific delivery (Garg et al. 2019).

2.4.2.1.2 Alginate

Alginates are one of the most versatile biopolymers with a range of uses (Smidsrod and Draget 1996). Alginic acid and its sodium and calcium salts are considered nontoxic and biocompatible (King 1983). Traditional use of alginate as a vehicle of medications varies depending on the thickening, gel-forming, and stabilizing properties. The need for sustained and enhanced regulation of drug use has grown the need for specialized polymers. Hydrocolloids, such as alginate, can play an important role in the structure of a sustained release substance. At a low pH, the hydration of alginic acid results in the development of a high-viscosity “acid gel.” Alginate is also readily gelled in the presence of a divalent cation, such as a calcium ion. Dry sodium alginate beads have re-emerged, producing a diffusion layer that limits the transport of small molecules (e.g. drugs). The tendency of alginate to shape two pH-dependent gel forms, i.e. an acid gel and an ionotropic gel, gives the polymer unique characteristics when compared to neutral macromolecules. The molecule can be tailored to a number of applications (Tonnesen and Karlsen 2002).

2.4.2.1.3 Xanthan Gum

Xanthan gum is a high-molecular-weight extracellular polysaccharide and hydrophilic polymer. It is an industrially essential polysaccharide that has a range of uses in various fields due to its distinctive rheological properties. This exopolysaccharide is formed by the bacterium *Xanthomonas campestris*, which uses glucose as the primary carbon source. Xanthan gum has been used as a thickening agent, binder, and disintegrate in medicinal formulations. Controlled release of drugs is important to increase the effectiveness of the drug delivery to the target area without creating a toxic impact. Xanthan gum has the ability to delay the release of drugs owing to its caking existence and the capacity to contain the drugs inside the gum (Benny, Varadarajan, and Ponnusami 2014). Xanthan gum not only slows down the release of medications but can also have time-independent release kinetics with additional stability and inertness advantages. Release of soluble drugs from this biopolymer happens mainly by diffusion, whereas barely soluble or insoluble drugs are released due to matrix erosion. It is also approved for use in both acid and alkaline media (Fernandez et al. 1993).

2.4.2.1.4 Cellulose

Cellulose is an important environmentally friendly renewable polymer (Meng et al. 2017). Cellulose has multiple functional groups on its base, which can be readily changed. Their hydrophilic sections consist of carboxyl (pectin), amino (chitosan), and hydroxyl (cellulose) groups which may form non-covalent bonds with biological tissues (Heinze and Liebert 2001). The molecular structure of cellulose confers significant characteristics such as hydrophilicity, chirality, degradability, and large chemical variation due to the high donor reaction of the OH groups (An and Hyeon 2009). Such qualities are essential features for use in drug delivery systems, such as large surface area and outstanding loading ability (Peres et al. 2016).

2.4.2.2 Liposomes

Liposomes are vesicles with their fundamental building blocks made up of phospholipids. Phospholipids consist of head and tail parts. The head is drawn to the water, and the tail, which is made up of a long chain of hydrocarbons, is repulsed by water. In nature, phospholipids are found in stable membranes consisting of two layers (a bilayer). Bilayer structures are referred to as liposomes, and monolayer structures are referred to as micelles. Liposomes are used for the distribution of drugs owing to their unique characteristics, including carting a wide range of hydrophilic and hydrophobic diagnostic or therapeutic compounds, having a higher load of drugs per molecule, and shielding encapsulated medicines from metabolic processes. The lipid bilayer arrangement may be changed to obtain certain desirable characteristics, including sustained circulatory half-life (stealth liposomes), the ability to form a nucleic acid complex to mediate gene transmission or genetic control, and the ability to deliver encapsulated cytosol material through the endosomal/lysosomal pathway. Common and stealth liposomes have the biological potential to increase the proximity of anticancer drugs on solid tumors (Daraee et al. 2016).

2.4.2.3 Dendrimers

Dendrimers are synthetic macromolecules that are strongly branched with conveniently modifiable surfaces. These structures are useful for functionalization and product conjugation. Their structure, which can be regulated by a variety of synthesis processes, allows for the regulation of characteristics such as shape, size, loading capacity, and solubility. The solubility and bioavailability of hydrophobic drugs can be improved. Drugs may be loaded into the dendrimers' intramolecular cavity or conjugated to their functional groups on the surface. The inclusion of functional groups in the dendrimer exterior often allows the introduction of additional groups that can actively target other diseases and enhance transmission, for example folate and antibodies, which are now commonly used as tumor-targeting strategies (Palmerston Mendes, Pan, and Torchilin 2017).

2.4.2.4 Inorganic/Metallic Nanoparticles

Inorganic nanoparticles like silver, gold, iron oxide, and silica nanoparticles have special characteristics such as SPR (surface plasmon resonance) which organic

nanoparticles do not possess (Patra et al. 2018). Inorganic nanoparticles are non-toxic, hydrophilic, biocompatible, and extremely stable (Paul and Sharma 2010). Drug delivery systems, designed to improve the safety of medications and adverse reactions, have evolved with the production of new materials. Binding of proteins, medicines, and other receptors are achieved through the alteration and functionalization of these nanoparticles with different functional groups (Thiruppathi et al. 2016). Medications may be conjugated to surfaces of nanoparticles by ion or covalent bonding as well as physical absorption and may be transferred and controlled by biological stimulus or light activation (Kong et al. 2017).

2.4.2.5 Nanocrystals

Nanocrystals are pure drug crystals of nanometer scales. Thanks to the advantages of high drug loading capacity, substrate durability, and ease of scale-up, nanocrystals have been commonly used to transport poorly water-soluble medicines (Lu, Li, and Wu 2016). Nanocrystals can be stabilized using polymeric substances or surfactants, which allow them to not use carrier groups. Clumping of nanocrystals in a reduced liquid solution is avoided by the introduction of a dispersing agent such as water or any aqueous or non-aqueous material, like liquid polyethylene glycol or oils. Nanocrystal characteristics also allow them to have enhanced saturation solubility, increased rate of dissolution, and increased adherence to surface/cell membranes. The approach used to synthesize nanocrystals is milling, high-pressure homogenization, and precipitation; however, refining is expensive due to the use and removal of organic solvents. The mechanisms by which nanocrystals promote the integration of a medication into the body involve enhancing solubility, the rate of suspension, and bioadhesion in the intestinal wall (Junyaprasert and Morakul 2015).

2.4.2.6 Carbon Nanotubes

There has been a strong interest in carbon allotropes with their potential applications in fields such as biosensing and imaging as well as high-strength and low-weight nanocomposite materials. Biological applications are made possible when these structures are functionalized with organic groups which make them soluble, nontoxic, and non-immunogenic. The high surface area of carbon nanotubes makes them ideal for the adsorption and conjugation of medicinal drugs as well as genes and proteins. Functionalization of carbon nanotubes often enables specific drug conjugation or targeting of tissues for treatment. Carbon nanotubes have needle-like structures which allow them to perforate cell membranes and so move through cellular components without causing apparent cell damage, resulting in drug carrier release inside the cell (Bianco, Kostarelos, and Prato 2005, Elhissi et al. 2012).

2.4.3 RECENT ADVANCEMENTS AND DISCOVERIES OF NANOMEDICINE

Advancements in nanotechnology-related drug delivery, treatment, and product development are beginning to change the medical environment. Site-specific

targeted distribution of medications and personalized medicine are just a few developments on the horizon. Progress has recently been made to show how nanomaterials can improve the bioavailability of natural substances *in vitro* and *in vivo*. Nanotechnology also showed the ability to alter compounds for drug targeting, tracking, and release. There are also many advantages of using nanotechnology to improve the production of non-synthetic compounds, but this is not without challenges. Drug delivery poses a problem, and the potential toxicity of nanomaterials needs to be further explored, especially if these systems are to be used for the treatment of chronic human diseases (Morrow, Bawa, and Wei 2007, Watkins et al. 2015).

Nanocarriers need to be optimized to realize the complete *in vivo* promise of nanomedicine in drug delivery. The fulfillment of this pledge is a clear understanding of both physiochemical and physiological processes. These are the foundation of the complex interactions found in the fingerprint of a nano-vehicle and its micro-environment. Sources involve carrier persistence, extracellular and intracellular drug release thresholds in various pathologies, encounters with the biological system, such as opsonization, and other obstacles on the route to the target site, whether physical, neurological, immunological, or biochemical, and the utilization of disease-state resources. Inherently, carrier configuration and targeting strategies differ depending on the type, stage of development, and position of the disease. Toxicity concerns are also of interest but are often overlooked. It is therefore important that computational modeling be carried out to solve these problems, if the effective use of these innovations is to be accomplished. The future of nanomedicine will rely on the reasonable layout of nanotechnology products and tools centered on a detailed and thorough comprehension of biological processes instead of forcing applications for certain materials popular today (Moghimi, Hunter, and Murray 2005).

2.5 CONCLUSION AND FUTURE DIRECTIVES

In turn, nanotechnologies seek to extend the scope of traditional disease classification and allow bedside diagnoses, the integration of diagnostics with therapeutics, and the development of customized medical care. The most significant therapeutic uses of currently available nanotechnologies are in the fields of biomarker discovery, cancer diagnosis, and identification of contagious microorganisms. The role of nano-biosensors is to actively monitor the accumulation of biomarkers for efficient disease diagnosis and effective disease management. It must also be a near-patient testing device, which requires a pure commitment to recognition. The ideal approach would be to add the sample to the sensing device and constantly monitor the amount of biomarkers present in the specimen (Prasad 2014).

The computational design of nanomaterials for biomedical imaging continues to improve and broaden. Highly effective molecular imaging contrast agents can be synthesized utilizing nanoparticles as their base. The nature of these probes has improved dramatically over the past decade, including multifunctionality, more effective tracking, greater biocompatibility, and agents tailored to each

accessible imaging modality. At the existing development stage, a nanomaterial contrast agent with the necessary attributes can be synthesized for any suitable use by various chemical processes which have been designed to manipulate the size and surface properties of nanomaterials for delivery, half-life, and removal performance. Nonetheless, there is room for significant improvements in biocompatibility, effectiveness, precision, and identification of new diseases; other issues include storage stability and long-term toxicology. In addition, modular nanomaterial systems that can be used for preclinical/clinical disease investigations need to be created. Finally, nanotechnology can continue to develop by producing new particles with novel and interesting characteristics for applications in medical imaging (Cormode et al. 2009, Nune et al. 2009).

Nanotechnology in medicine is likely to have a substantial effect on human quality of life by bettering the delivery of drugs and treatment of diseases. Nanomedicine typically involves encapsulated therapeutic compounds in nanoscale materials. Nanomedicine is typically designed to increase the therapeutic index of substances by facilitating effective distribution to the target site and enhance therapeutic efficacy and/or decrease retention at normal body sites to mitigate toxicity. Nano-encapsulation can also shield compounds from deterioration of biological conditions and can provide solubilization (Patra et al. 2018). Experimental production of nanomedicines continues to progress at a rapid pace, but significant challenges remain in the advancement of these technologies for clinically viable therapies. These problems include the detection of *in vivo* bio-distribution of nanoparticles following administration in animals and humans, characterization, the creation and evaluation of innovative and functional methods capable of identifying possible short- and long-term health hazards, biocompatibility, large-scale manufacturing, and governing laws (Hua and Wu 2018). Continued translational progress will therefore entail coordination and cooperation between specialists engaged in all phases of nanotechnology pharmaceutical production, including pharmaceutical design and manufacturing, cellular interactions and toxicology, and pre-clinical and clinical evaluations.

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3 Nanomaterials for Drug Delivery

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3.1 INTRODUCTION

Nanotechnology is a skill of synthesis of nano-size-ranged materials with numerous benefits over bulk materials. Nanotechnology is employed in different areas such as in medicine and agriculture. In the medical field, since the 1990s, nanotechnology is being researched and practiced as a method of improved drug delivery options for tissue engineering, disease screening, and diagnosis because it provides numerous advances over conventional therapeutics [1–3]. Nanotechnology provides a possible option to engineer the bulk material to the nanoscale with significant physical, optical, chemical, and electrochemical properties.

The smart nanostructured materials present various advantages such as enhanced systemic bioavailability, improved stability, controlled release, and targeted delivery of therapeutics to a specific site, which in turn reduced the dose

and side effects of therapies and exhibited safe and effective drug delivery [4]. Additionally, the small size favors passive targeting by enhanced permeation and retention (EPR) effect for cancer cell targeting [5]. Further, such nanomaterials can provide better permeation through physiological membranes. Nanomaterials have been investigated for oral, topical, ophthalmic, and parenteral deliveries. Different nanocarriers have been studied for effective delivery of genes- and peptide-based therapeutic agents in disease conditions such as cancer, skin disorders, rheumatic arthritis, central nervous system disorders, ocular diseases, and other infectious diseases. These studies demonstrated the improved therapeutic outcomes compared to drug delivery systems [6–8]. Figure 3.1 represents the application of nanomaterials in various biomedical area.

For the preparation of nanoparticles, various organic and inorganic materials are used, including proteins, lipids, metals, polymers, and clays. Based on the material of preparation, nanomaterials are classified into different types like polymeric nanoparticles, nanocrystals, liposomes, micelles, solid–lipid nanoparticles (SLNs), carbon nanomaterials, and inorganic nanoparticles [9]. Table 3.1 presents the types of nanoparticles employed in drug delivery and a list of core materials used for preparation [10–13]. The classification of nanomaterials based on the type of materials is illustrated in Figure 3.2. These have been explained in

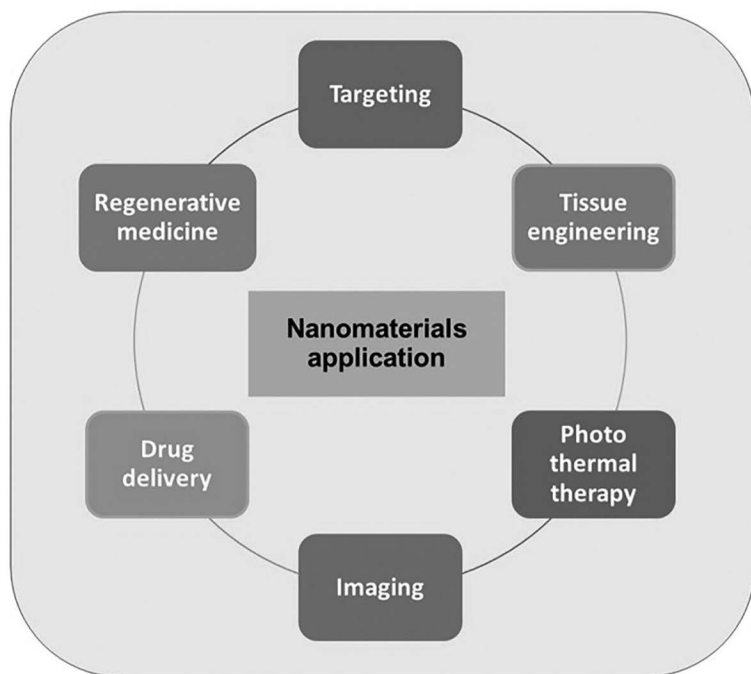


FIGURE 3.1 Applications of nanomaterials in the biomedicine field.

TABLE 3.1**Various Nanoparticles Employed in Drug Delivery and a List of Core Materials Used for Preparation**

Nanoparticles	Materials Used
Polymeric nanoparticles	Synthetic polymers —poly(lactic acid) (PLA), poly(lactic-co-glycolide) (PLGA) copolymers and polycaprolactone
Liposome	Natural polymers —Xanthum gum, alginate, gelatin, zein, chitosan, albumin Phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, 1,2-dilauroyl-sn-glycero-3-phosphocoline (DLPC), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (sodium salt) (DOPS), dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dipalmitoylphosphatidylseine, dipalmitoylphosphatidylglycerol, 1,2-dilauroyl-sn-glycero-3-phosphocoline (DLPC)
Solid lipid nanocarrier	Carnauba wax, glyceryl monostearate, glyceryl dibehenate (Compritol®888 ATO), cetyl palmitate (Crodamol™ CP), glyceryl palmitostearate (Precirol ATO®5), trimyristin (Dynasan®)
Nanostructured lipid carrier	Solid lipids —carnauba wax, glyceryl monostearate, glyceryl dibehenate (Compritol®888 ATO), cetyl palmitate (Crodamol™ CP), glyceryl palmitostearate (Precirol ATO®5), trimyristin (Dynasan) Liquid lipids —Labrafil M1944, Labrafil M2125, Labrafac cc™, Miglyol®812 and 810, Softison 378, oleic acid, lauroyl polyoxyglycerides (Gelucire®44/14)
Liquid crystalline nanoparticles	Phytantriol, oleyl glycerate, phytanyl glycerate, glyceryl monooleate, lecithin, hydrogenated castor oil
Dendrimers	Polyamidoamine (PAMAM), polypropyleneimine (PPI), poly(esteramine) (PEA), poly-L-lysine, melamine, poly(etherhydroxylamine) (PEHAM)
Carbon nanomaterials	Graphene, carbon nanotubes, carbon nanohorns, nanodiamonds
Polymeric micelles	Diblock(poly{lactic acid}–poly{ethylene glycol}), triblock(poly{ethylene oxide}–poly{propylene oxide}–poly{ethylene oxide}), poly(ethylene glycol) (PEG) and polymerized phenylboronic ester-functionalized methacrylate (PBEMA)
Inorganic type of nanomaterials	Magnetic nanoparticles (iron, nickel, and cobalt), halloysite nanoclay nanoparticles (mineral silicates), silver nanoparticles, gold nanoparticles

detail with regard to their characteristics, properties, and applications. Based on the delivery systems' requirement, these nanoparticles are being used centered on their properties and structure.

In the current clinical market, many nanomedicines have got approval and many others are in clinical trials for different indications. Nanomedicine preparation is a complex and time-consuming process compared to conventional medicines. The key challenges in the development of nanoparticle-based medicines include large-scale manufacturing, reproducibility in characteristics, biological challenges, biocompatibility and safety issues, regulatory agency's requirement, intellectual property-related challenges, and cost-effectiveness.

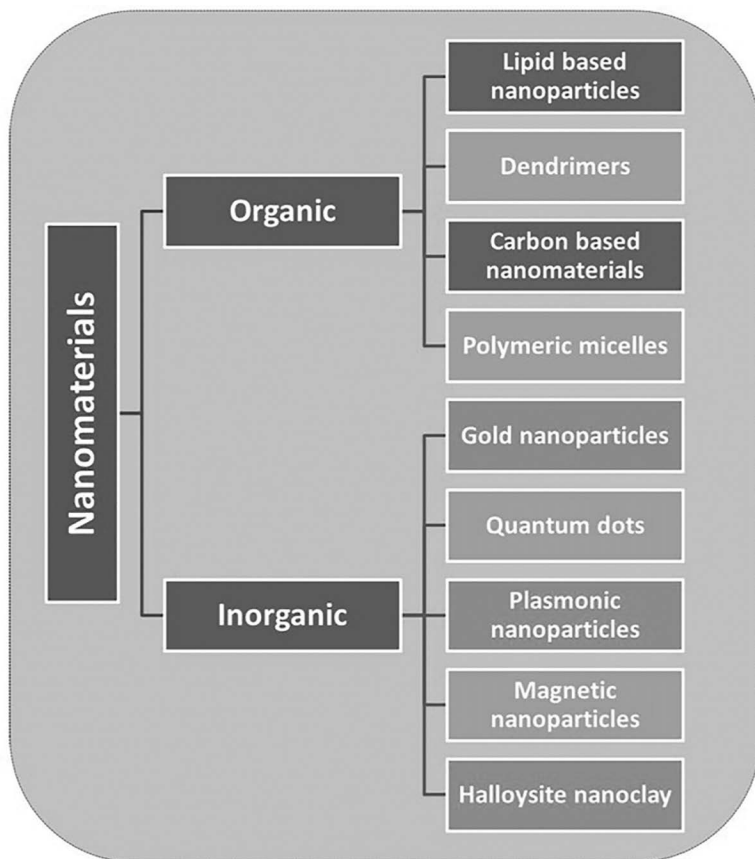


FIGURE 3.2 Classification of nanomaterials based on the type of materials used.

All these challenges and issues act as major barriers for nanomedicines to appear in the market to the expected extend [14–16].

3.2 CLASSIFICATION OF NANOMATERIALS

3.2.1 ORGANIC TYPE OF NANOMATERIAL

Organic nanomaterials have been growing in the field of pharmaceutical and bio-medical applications like drug delivery, wound healing, bone regeneration, skin and tissue regeneration, and many more because of its advantages of natural and organic structures. For the preparation of organic nanoparticles, carbon-based nanomaterials and biodegradable polymers such as chitosan, alginate, xanthan gum, poly(lactic-co-glycolic) acid (PLGA), and silk fibroin can be used [17]. They give a controlled drug delivery [18,19] and reduced side effects with other promising results for bioavailability. Many different types of organic nanoparticles

that have been developed are described in the following parts with their physico-chemical properties and applications. Table 3.2 presents the comparative description of nanocarriers with focus on their architecture, drug position, benefits, and limitations [13,20–25].

3.2.1.1 Lipid-Based Nanoparticles

Currently, many different drug delivery systems have been coming into emergence, among which nanoparticulate systems prepared from lipids are growing very fast. Natural and synthetic lipids and oils are used to prepare nanoparticles to increase drug permeability. They can also help in overcoming the first-pass metabolism, resulting in the enhancement of oral bioavailability. The lipids used in the formulation are inert, non-toxic, and biocompatible. Purposely, lipid-based nanoparticles are used to enhance the absorption of drugs by different mechanisms, including enhancing solubilization, avoidance of precipitation of the drug on dilution in body fluid, improving permeability, and blocking efflux transports [11,26]. Lipid-based nanoparticles include liposomes, SLNs, and nanostructured lipid carriers (NLCs), which have been described in detail in the following sections.

3.2.1.1.1 *Liposomes*

Liposomes are phospholipid bilayered vesicles composed of natural and synthetic phospholipids along with cholesterol surrounding the distinct aqueous core [27]. The vesicular systems consisting of natural and synthetic lipids have been presented as a versatile nanomaterial platform for the development of a novel drug delivery system in a wide scope of biomedical applications. Because of the presence of both aqueous and lipid compartments, liposomes are capable of entrapping both hydrophobic and hydrophilic drugs in the lipid membrane and aqueous compartments, respectively. For medicinal use, liposomes in the size range of 50–450 nm are preferred [28]. Liposomes have structural similarity to that of cellular membranes, which makes it a most successful system for drug delivery [27].

The efficacy of liposomal formulation solely depends on the physicochemical properties of the membrane, i.e., its size and charge on the nature of its components and its organization. The lipid membrane of the liposomes is composed of phospholipids, which are amphiphilic molecules having a polar head with a non-polar tail. In aqueous solutions, these phospholipids tend to form two lipid layers due to the tendency of the head to interact with the aqueous phase, while the tail has a tendency to interact with one another. The addition of cholesterol to the lipid bilayer membrane decreases the fluidity that improves the stability because of its dense packaging with phospholipids. In both the lipid layers, hydrophobic portions face each other forming a hydrophobic compartment acting as a barrier for the permeability of materials, both inside and outside. Van der Waals' interactions between hydrophobic tails, hydrogen bonding, and polar interaction between polar head and water molecules stabilize the liposomal organization [29].

Depending upon the method of preparation, size, and structure, liposomes can be classified into different types. Unilamellar vesicles (ULVs) are capable of

TABLE 3.2
Comparative Descriptions of Nanocarriers with Focus on Their Architecture, Drug Position, Benefits, and Limitations

Nanocarrier	Drug Attachment	Architecture (Focuses on Material)	Benefits	Limitations
Liposome	Encapsulated hydrophilic drug in core and hydrophobic drug into phospholipid bilayer	Self-assembled phospholipids in bilayers	Biocompatible, high drug loading, and non-toxic	Very high production cost Leakage of encapsulated drug
Solid-lipid nanoparticle	Hydrophobic drug encapsulated within the solid-lipid core	Solid-lipid core matrix	Cationic liposomes can be successfully used for gene and nucleotide delivery No biotoxicity	Low drug-loading capacity Long-term storage leads to drug expulsion
Nanostructured lipid carriers	Encapsulated into the crystalline lattice	Blend of solid and liquid lipid as core matrix with crystalline imperfections	High drug-loading capacity Impediments drug expulsion	Cytotoxic effects associated to the nature of matrix and concentration
Liquid crystalline nanoparticles	Both hydrophilic and hydrophobic drugs encapsulated into the core	Partially ordered, anisotropic fluids	Adaptable for drug and gene delivery Enhanced in vivo stability Increased drug loading	Low entrapment efficiency for hydrophilic drugs
Dendrimers	Embedded into the polymer branches	Highly branched star-shaped polymer matrix	Explored for peptide and protein delivery Effective for gene delivery	Multiple steps involved in synthesis
Carbon nanotubes	Drug linked onto the carbon backbone	Hollow graphitic cylindrical tubular structure with hexagonal arrangement	Effective to deliver small drugs and proteins Increased drug loading	Cytotoxicity Accumulation in body Poor biocompatibility

(Continued)

TABLE 3.2 (Continued)
Comparative Descriptions of Nanocarriers with Focus on Their Architecture, Drug Position, Benefits, and Limitations

Nanocarrier	Drug Attachment	Architecture (Focuses on Material)	Benefits	Limitations
Polymetric micelles	Hydrophobic drugs embedded in to the hydrophobic core	Spontaneous arrangement of amphiphilic block copolymers in aqueous solutions	Biocompatible with lowered toxicity and reduced side effects	Undergoes deformation and disassembly; thus lacks controlled drug release
Quantum dots	Linked on to the surface	Nanocrystals/particles of a semiconducting material	Multiple molecular targets	Metal core causes toxicity
Gold nanoparticles	Linked to the surface	Face-centered cubic structure with gold	Non-invasive	Poor biocompatibility
Magnetic nanoparticles	Linked to the surface or surface coating	Iron oxide core enclosed by biocompatible coating	Enhanced contrast Multiple molecular targets Low cytotoxicity	Lack of scale-up ability due to high cost
Halloysite nanoclay	Drug loaded onto the surface	1:1 Al:Si ratio of aluminosilicate in two layers (montmorillonite)	Innate magnetic properties Recognized as a gene delivery agent for siRNAs, oligonucleotides, and DNA	Poor aqueous dispersion ability Lack of human studies for pharmaceutical application

enclosing hydrophilic drugs as they hold a large aqueous compartment. In contrast, multilamellar vesicles (MLVs) have two or more lipid bilayers, which makes it a worthy candidate for entrapment of hydrophobic drugs. The release characteristics of the drug changes in ULVs and MLVs depending upon the number of lipid bilayers.

Liposomes are considered to be an appropriate delivery system due to their biocompatibility, biodegradability, ability to entrap both hydrophobic and hydrophilic drugs, protection of the active drug from enzymatic or chemical degradation, and reduction in undesirable side effects with desirable cytotoxic effects at tumor sites [30].

Jhaveri et al. prepared a resveratrol-loaded liposomal (RES-L) formulation to target transferrin receptors for the treatment of glioblastoma (GBM). Mostly, transferrin receptors are up-regulated in GBM, so the liposomes were modified with transferrin moieties (Tf-RES-L) to target cancer cells. The prepared liposomes were found to be stable with good drug-loading capacity and exhibited controlled release of drugs in *in vivo* conditions. The efficacy of prepared Tf-RES-L formulation was examined in a subcutaneous xenograft mouse model of GBM, which proved effective in tumor inhibition than other treatments and increased survival in mice. This study favors the use of liposomal formulations for tumor targeting [31].

In other research, the effect of liposomal formulation on the bioavailability of drugs was examined. In this study, glucosylated liposomes were designed for the delivery of usnic acid for handling bacterial infections. The biological activity of cationic glucosylated usnic acid liposomes was evaluated against biofilms based on *Staphylococcus epidermidis*. The results revealed that cationic nature promotes penetration of liposome into the biofilm, which in turn increased the antimicrobial activity [32].

3.2.1.1.2 Solid-Lipid Nanoparticles (SLNs)

SLNs are lipid-based nanoparticles having solid-lipid core with a size range of around 50–1000 nm after the drug incorporation. For solid core, triglycerides, waxes, or glyceride mixtures are used as these are solid at room temperature and body temperature as well [33]. To improve the stability of these nanoparticles, surfactants are also added to the formulations which coat these SLNs. The drug in the formulation is either dispersed or dissolved in the solid-lipid core matrix. These are similar to oil in water nanoemulsion, where the liquid lipid is replaced with solid lipid giving rise to SLNs. There are various techniques for their preparation, among which cold homogenization, hot homogenization, and emulsification solvent evaporation are the most commonly used [34,35].

Drug delivery through SLNs contributes to various advantages, including improved bioavailability, non-toxic behavior, suitable for incorporation of hydrophilic and lipophilic drugs, and biocompatibility along with enhanced stability against drug leakage, biodegradability, hydrolysis, and coalescence. SLNs can improve the *in vivo* performance of drugs and thus can decrease the dosing frequency [36]. Other advantages of SLNs are low cost, large-scale production, and

they do not require organic solvents for the preparation. Additionally, powdered form of SLNs can be loaded into capsules, pellets, or tablets to further improve drug delivery [37]. In SLNs with solid–lipid core, mobility of drugs get decreased, which enhances the controlled release of the loaded drug.

Vijayanand and his team prepared herbal extract of *Hibiscus rosa sinensis*-loaded SLN (HSLN) using glycerol monostearate and beeswax. The prepared HSLN was evaluated for its activity against depression in Swiss albino mice. The animal groups were treated with different doses of HSLNs (10 and 50 mg/kg) and crude extract (200 mg/kg). The results exhibited that the designed SLNs showed similar pharmacological activity at 20 times lower dose compared to crude extract [38].

The improved dissolution and bioavailability properties of SLNs have been confirmed in a study of ezetimibe-loaded SLNs. The dissolution studies illustrated that ezetimibe-loaded SLNs exhibited a higher solubilization profile compared to the drug suspension and marketed product. The pharmacokinetic studies demonstrated that ezetimibe-loaded SLNs showed 3 and 16 times improved bioavailability when compared to marketed product and drug suspension, respectively [39].

3.2.1.1.3 Nanostructured Lipid Carrier (NLCs)

The advantages of SLNs have been described in the earlier section, which has confirmed its superiority and advancement over liposomes. There are some disadvantages associated with SLN as these are perfectly crystalline lipid materials in which loaded drugs can lodge. During production and storage of the formulation, this crystalline behavior results in expulsion and burst release of loaded drug. Further, the next-generation nanoparticles NLCs have been grown and emerged as suitable carriers for the delivery of therapeutic substances [40].

In contrast to SLNs, NLCs have an amorphous or imperfect crystal structure of the lipid matrix, which in turn permits the drug to get loaded into the lattice in molecular form as well as aggregates of a bunch. Thus, NLCs can load more amount of drug with less probability of drug expulsion [41].

NLCs are nanomaterials of the size range around 100–500 nm consisting of a blend of solid and liquid lipids as a solid–lipid matrix along with an aqueous phase having a surfactant or a mixture of surfactants. These solid–lipid matrix and surfactants are combined in different ratios and combinations [42]. Among several preparation methods of NLCs, high-pressure homogenization is preferred because here no solvent is required for the preparation. These delivery systems can enhance the encapsulation efficiency and drug loading with improved physical stability and controlled release profile of the loaded drug. The solid–lipid matrix in NLCs acts as a physical barrier that protects the encapsulated drug compound from degradation and damaging factors as well.

Depending on the formulation components and the production parameter employed, three different types of NLC structures can be obtained, namely, imperfect-type, amorphous-type, and multiple-type NLCs. Imperfect-type NLCs can be obtained by mixing solid lipids with chemically different liquid lipids or oils.

The amorphous-type NLCs can be obtained by mixing solid lipids with distinct lipids like isopropyl myristate or hydroxy octacosanyl hydroxy stearate. In multiple-type NLCs, little lipid nano-compartments of oil are present in the solid matrix [43].

In a study, lopinavir-loaded NLCs were formulated using a high-shear homogenization method. The NLCs were spherical with a size range of 286.8 ± 1.3 nm. The in vitro cell uptake studies and in vivo bioavailability studies were conducted on the Caco-2 cell line and male Wistar rats, respectively. The results demonstrated that lopinavir-loaded NLCs improved cell uptake and 6.98 times higher bioavailability compared to lopinavir suspension [44].

Chakraborty et al. studied NLCs for the delivery of zidovudine to the brain. The designed zidovudine-loaded NLCs exhibited the size range of 54.7 ± 1.4 nm and sustained release behavior on zidovudine in the cerebrospinal fluid. In vivo studies of NLCs in the rat showed improved brain permeation with high mean residence time, C_{max}, and AUC. This study also confirmed the localized delivery of zidovudine with NLCs accumulation in the brain. Conclusively, the results suggested that NLCs can be a potential delivery system for the long-term delivery of zidovudine in the brain for the treatment of acquired immunodeficiency syndrome [45]. Rapalli et al. prepared curcumin-loaded NLCs formulation for enhanced skin-retained topical drug delivery. The results of ex vivo skin permeation studies indicated that prepared curcumin NLCs showed a 3.24-fold increased permeation and skin retention compared to free curcumin gel. Thus, NLCs have the potential to treat various chronic inflammatory conditions on topical application [46]. Utilizing the NLCs for topical drug delivery protects the drug from degradation and enhances permeation by the occlusive effect. Further, the NLCs hydrate the skin, exhibit controlled release of the drug, and impart local action for prolonged time (Figure 3.3) [11].

3.2.1.1.4 Liquid Crystalline Nanoparticles (LCNPs)

Liquid crystalline nanoparticles (LCNPs) are non-lamellar nanoparticles that are developed by dispersion of self-assembled liquid lipids in excess of water [27]. Nowadays, these LCNPs are determined as a promising strategy for the delivery of drugs. These formulations are suitable for the delivery of drugs, proteins, and peptides, as these can encapsulate both hydrophilic and lipophilic molecules. For the preparation of LCNPs, crystalline lipids and stabilizers are required for the formulation of nanostructures and stabilization of dispersions, respectively [47]. When lipids are dispersed in an aqueous phase to get stable reverse phases, the stabilizer is added to reduce the interfacial tension by reducing the interaction between hydrophobic and aqueous forms. This, in turn, prevents the aggregation of the dispersed phase. The lipid used in the formulation should offer a stable reversed-cubic structures reversed hexagonal, sponge, reversed micelles [48,49]. Various types of amphiphilic lipids such as phytantriol, phytanyl glycerate, glyceryl monooleate, oleyl glycerate, lecithin, silicone oil, and hydrogenated castor oil are used for the preparation of LCNPs, and because of their internal structures, they provide different kinds of crystalline phases. Figure 3.4 depicts the types of

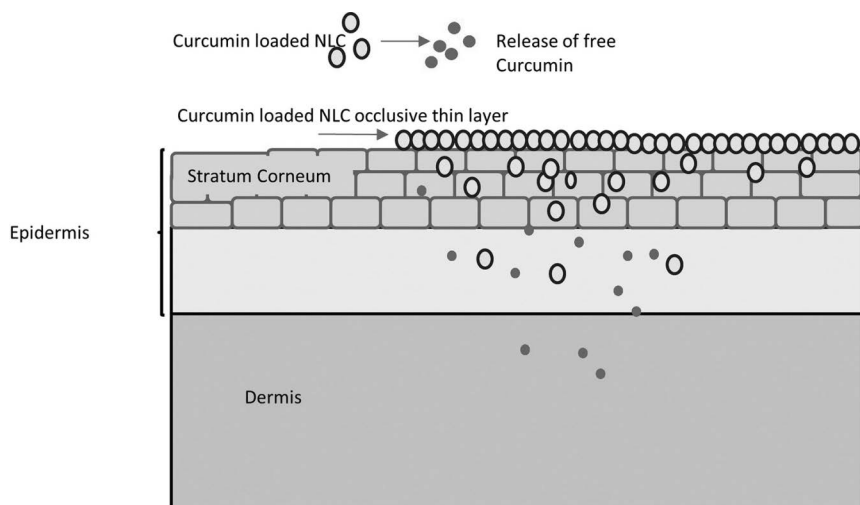


FIGURE 3.3 The permeation of curcumin-loaded NLC into the skin due to formation of thin film by NLC formulation. The three major layers of skin such as stratum corneum, epidermis, and dermis. Penetration of curcumin-loaded NLC through the skin and its release of curcumin in skin layers. This shows occlusive nature and promotes the permeation of NLC into stratum corneum. (Reprinted with permission from Rapalli VK et al., *Spectrochimica Acta. Part A: Molecular and Biomolecular Spectroscopy*, Elsevier 224, (2020):117392.)

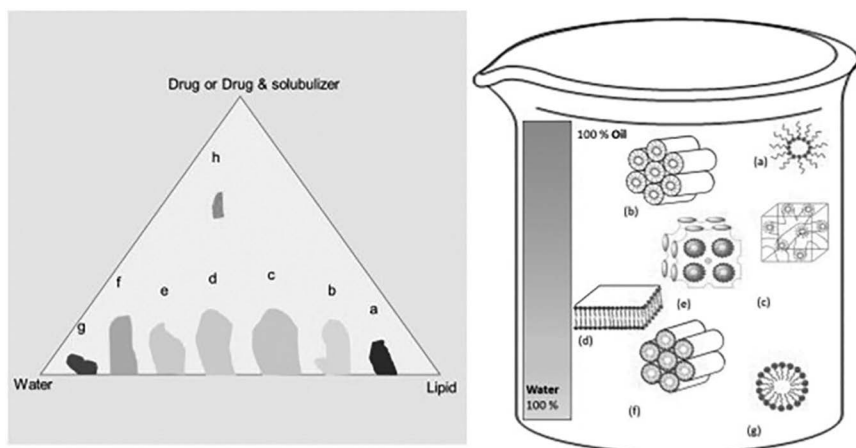


FIGURE 3.4 Various types of LCNPs (left), and the ternary-phase diagram (right) presents different phases: (a) reverse micelles, (b) reverse hexagonal, (c) reverse bicontinuous cubic, (d) lamellar, (e) normal discontinuous cubic, (f) hexagonal, (g) normal micelles, and (h) microemulsion. (Reprinted with permission from Rapalli VK et al., *Journal of Molecular Liquids*, Elsevier 315, (2020):11377.)

LCNP and the ternary phase diagram presents the various phases [20]. The stabilizer added in the formulation should be amphiphilic so that it does not interfere with the crystalline lipid phase [50].

With LCNPs, higher drug encapsulation can be accomplished because of the presence of higher lipid concentration in the formulation along with higher open area. The characteristics of LCNPs include high drug payload, broad solubilization spectrum, bioadhesive nature, non-toxic potential, sensitive drug stabilization and protection, prolonged residence time, and sustained release profile [51]. Other than these, the physical and chemical structures of LCNPs provide great accessibility to modify its interfacial properties by varying and striking the desired functionality at the surface [49]. The main applications of LCNPs are matrices for the crystallization of proteins, biomimetics, and as drug delivery vehicles.

Along with increasing the residence time, LCNPs also have the potential for improving bioavailability, which was demonstrated in a survey led by Rui Liu and co-workers. In this study, tetrandrine-loaded LCNPs were prepared for ocular delivery using GMO, poloxamer 407, and water as primary components and Gelucire 44/14 and amphipathic octadecyl-quaternized carboxymethyl chitosan as secondary components. The formulations were optimized using central composite factorial design and characterized for *in vitro* and *in vivo* parameters. Fluorescence imaging characterization was opted to analyze the pre-ocular retention and microdialysis for finding out the pharmacokinetic behavior. The study results demonstrated a particle size of 170 nm with a high encapsulation efficacy of 95.46% and enhanced pre-ocular retention by 2.03 folds compared to tetrandrine solution. The pharmacokinetic studies in rabbits revealed that tetrandrine LCNPs exhibited 2.65 times higher ocular bioavailability compared to tetrandrine solution [52].

3.2.1.2 Dendrimers

Dendrimers are nano-sized radially symmetric polymeric macromolecules having a tree-like branched structure comprising of the focal core, a peripheral layer containing different functional groups, and an interior layer having several building blocks for the formation of branches [53]. These are homogeneous, monodisperse molecules containing symmetric branching units around a small molecule [54]. The shape of polyionic dendrimers is not persistent and may experience changes in size, shape, and flexibility as there is an increase in generations. The functional groups present at terminal positions of dendrimers determine the efficiency of drug entrapment and complexation of nucleic acid with dendrimer. On the outer surface, the presence of highly reactive and interactive groups makes the molecule highly reactive, soluble, and also improves the binding properties [55].

Dendrimers are nanomaterials that have limited structural diversity compared to other nanomaterials. But, dendrimers have the power to enhance the solubility and bioavailability of water-insoluble drugs, modify the pharmacological properties by forming a covalent bond with the small drug particles, and form polymeric scaffolds. Moreover, the self-assembling property, electrostatic

interactions with oppositely charged molecules, and polyvalency help to develop multiple interactions with biological receptors. All these are crucial to the novel drug delivery technology; hence, dendrimers have been successfully used in medical fields [56].

On the other hand, the morphological features of dendritic polymers are similar to that of proteins, enzymes, and viruses, which makes them suitable for biomedical applications [57]. Other than these, dendrimers are emerging as a novel delivery system for drug delivery to tumor sites in cancer patients [58]. These are used for transdermal drug delivery, gene delivery, as a contrast agent [59], for improving the solubility properties of drugs, in photodynamic therapy [60], and in burn treatment.

In a recent study, researchers developed a dendrimer by conjugating generation 4 polyamidoamine (G4 PAMAM) with polyethylene glycol (PEG)–phospholipid copolymer which was then loaded with siRNA on PAMAM moieties and sparingly soluble chemotherapeutic agents into the hydrophobic core. This formulation was developed to downregulate the multidrug resistance (MDR)-related proteins to reverse the MDR effect. The prepared dendrimer was co-loaded with DOX and therapeutic siRNA, and was then tested for its cytotoxic effect against MDR tumor cells. They reported that the combination successfully downregulated P-glycoprotein in MDR tumor cells and ultimately reversed the resistance, which was developed against DOX. Additionally, the use of PAMAM dendrimer facilitated the complexing of siRNA along with cellular interaction and also assisted endosomal escape [61].

The modification of dendrimers can be advantageous beyond dendrimers alone. This property was exploited by one group of scientists where they explored modified PAMAM dendrimers as a drug delivery system. They adopted the concept of grafting carboxyl-terminated PAMAM G3.5 with poly(ethylene glycol) methyl ether (mPEG) to overcome the issues of toxicity and premature clearance of full generation dendrimers. Anticancer drug carboplatin has been loaded into the modified PAMAM dendrimers. The particles were found to be spherical with a mean particle size of 36 nm. The neutral charge on surface nanoparticles proved that the prepared formulation was cytocompatible. The 24-hour *in vitro* release pattern verified the prolonged-release pattern of carboplatin compared to the free drug [62].

3.2.1.3 Carbon Nanomaterials

Carbon nanomaterials are the most extensively studied nanoscaled materials because of their unique physical and chemical properties, i.e., mechanical, thermal, electrical, and optical properties. Due to these attributes, carbon nanomaterials have been widely explored in biomedical applications, and their uses have been increasing for the treatment of various kinds of diseases [63]. Preferentially used carbon-structured nanomaterials are graphene, carbon nanotubes, carbon nanohorns, and nanodiamonds. These carbon nanomaterials have different characteristics that make them a suitable system for drug delivery. Carbon nanomaterials can disperse in aqueous solution and drugs can be extensively loaded

on their surface by covalent or non-covalent bonding or in the internal cores of carbon nanostructures [64]. Carbon nanomaterials can translocate through cellular membranes and have the capability to enter the cells via energy-dependent endocytic pathways. Further, these can aggregate in cancer tissues using EPR effect [65]. Along with drugs, some targeting materials and imaging tags, such as fluorescent probes and radioactive nuclides, can also be incorporated or linked to these nanomaterials for non-invasive targeting and observing the intracellular environment, respectively. The functionalized nanotubes can easily cross the blood–brain barrier (BBB) because of its nano-dimensions and needle-like morphology [24].

The graphene is a hydrophobic molecule, and its oxidized form graphene oxide is mainly used for drug delivery. The graphene oxide with hydroxyl and carboxylate groups can covalently bind with various drugs under mild conditions. DOX and camptothecin were studied with graphene for their effective delivery. For further stabilization, graphene oxides are employed with water-soluble and biocompatible polymers like PEG [66].

Carbon nanotubes are a unique kind of nanomaterials having a tubular, hollow monolithic structure with the ability of multifunctional surface chemistry which ultimately provides them the capacity of high drug loading, biocompatibility, reduced immunogenicity, and photo-luminescent activity [24]. Due to all these advantageous features, carbon nanotubes are becoming the area of focus for drug delivery, tissue engineering, and imaging purposes. These carbon nanotubes can be further modified by carboxylation, esterification, amidation, PEGylation, and polymer wrapping, which in turn improves the properties of the designed drug delivery. For research purposes, carbon nanotubes are mostly used for the delivery of anticancer drugs, and other than this, it is also used in the study of antimicrobial agents [67].

Carbon nanomaterials are designed for the development of a sustained and controlled delivery system. By modifications with proteins or magnetic nanoparticles, specificity of a molecule related to the present disease can be achieved. There are many pieces of research where these nanomaterials have been used as programmed and controlled drug delivery systems [68]. Carbon nanoparticles are also able to provide stimuli-responsive triggered release with controlled release of the drug [69].

Bhirde and his co-workers conducted a research study to confirm the cellular targeting behavior of these nanomaterials. For this purpose, they attached cisplatin and epidermal growth factor (EGF) to single-walled carbon nanotube for targeting squamous cells specifically. In vitro imaging of the neck and head squamous carcinoma cells showed that these carbon nanotubes were internalized rapidly into the malignant cells. For imaging, it had been injected into mice, and video imaging revealed the selective uptake of single-walled carbon nanotubes containing cisplatin and EGF by head and neck squamous cells and confirmed rapid regression of tumor growth [64].

To research the possible applications of multi-walled carbon nanotubes (MCNTs) for colon-specific drug delivery via an enzyme-responsive mechanism,

many studies have been accompanied. In one such study, an enzyme-sensitive colon-specific tablet (PM tablet) was devised with the complex of pectin and MCNT using the wet granulation method [70].

In a study, *in vitro* drug release using PM tablet in a non-enzymatic environment showed less than 30% release in 6 hours, but in the presence of pectinase enzyme, there was degradation of release. In this, the drug was incorporated into MCNTs, and then this was embedded into pectin. Therefore, PM tablets successfully prevented the leakage of MCNTs in the gastrointestinal tract. Thus, this can be a promising colon-specific drug delivery system.

3.2.1.4 Polymeric Micelles

Polymeric micelles are a core-shell type of a nanoparticulate system formed spontaneously in the aqueous system by amphiphilic copolymers when the concentration of this copolymer is more than its critical micelle concentration (CMC) [71]. To form these copolymers, multiple unimers are linked chemically to form block copolymers. These unimers consist of hydrophobic and hydrophilic portions which cause the difference in the solubility of the segments in the aqueous solution. Due to this solubility difference, micellization occurs spontaneously, polymers associate, and micelles are formed. The morphology of this micellar system is the lowest energy spherical confirmation at body temperature in the range of 10–100 nm. The stabileness of the system is specified in terms of its power to stand dilution. CMC of the polymer describes its ability to remain stable on dilution, because below CMC level, micelles dissociate into unimers. Therefore, it can be said that the lower the CMC, more stable the micelle and more the capability to withstand the dilution [72].

Generally, for the formation of polymeric micelles, amphiphilic di-block, triblock, and graft copolymers are used. The drug in these polymeric micellar systems can be either encapsulated physically inside the core portion or can be chemically attached. Further advanced copolymers with pH, temperature, ultrasound, enzyme, and light-responsive properties have emerged, which in turn provide controlled dissociation of micelles and triggered drug release in response to specific stimuli [73]. The chemical covalent bonding stabilizes the drug more than physical encapsulation and also controls the release characteristics of the system. Three types of drugs can be entrapped between the core and the shell part in this system: hydrophobic or non-polar drug inside the core part, hydrophilic or polar in the shell portion, and drugs with intermediate polarity [74].

Polymeric micelles can be considered advantageous for drug delivery purposes because of their properties like biocompatibility, solubilization of poorly soluble drug molecules, structure, size, core-shell architecture, low toxicity profile, dissociation, controlled release, enhanced retention time, and high stability [75].

Due to the characteristics and advantages over other available formulations, polymeric micelles are very much studied for various diseases like anti-influenza therapy, estrogen therapy, and especially for cancer tissue targeting. Recently, Wang and the team prepared methotrexate-loaded polymeric micelles using

monomethyl poly(ethylene glycol)–poly(ϵ -caprolactone) (MPEG–PCL) as the copolymer. These prepared methotrexate-loaded micelles demonstrated a particle size of 25.64 ± 0.99 nm with an encapsulation efficacy of $92.46\% \pm 2.38\%$. In vitro examination revealed the slow and sustained release of drug from micellar formulation compared to free drug. The methotrexate-loaded micelles were found to be more effective in inhibiting cellular propagation and inducing apoptosis in Raji lymphoma cells compared to high-dose methotrexate injection. Cellular uptake of these micelles was found to be at 1.5 times higher rate in Raji cells compared to free drug. From these results, they concluded that MPEG–PCL-methotrexate micelles were advantageous over intravenous formulation for enhancement of solubility and anti-tumor activity, which can meet the clinical demand more effectively [76].

Stimuli-responsive drug release with controlled-release activity is very important in terms of drug delivery [77]. But for the development of such formulations, the amount of drug loading is a major issue. Wang and colleagues developed a micellar system using a copolymer that responds to the stimulus of reactive oxygen species and esterase (dual responsive polymeric micelles). The synthesized copolymer successfully self-assembled into a micellar system that could load 6.99% drug with an entrapment efficacy of 76.9%. This study confirmed the stimulus-responsive controlled release profile with increased drug loading without increasing the amount of drug. The designed micellar system showed a good stability profile with lower CMC and confirmed long circulation time in the body [78].

3.2.2 INORGANIC TYPE OF NANOMATERIALS

An inorganic nanoparticle is a kind of nanomaterials made up of inorganic raw materials like carbon, gold, silica, silicon, iron, etc. These particles have properties of non-toxicity, hydrophilicity, higher stability, and biocompatibility. Its non-immunogenic behavior and cell uptake ability have grabbed the attention of many researchers in this field. The size, shape, and characteristics of these nanoparticles are greatly influenced by the electromagnetic, optical, and catalytic properties of the metal nanoparticles. The ability to resist microbial attack is another advantage of these inorganic nanomaterials, which makes them extremely stable. Table 3.2 summarizes the comparative description of nanocarriers with focus on their architecture, drug position, benefits, and limitations [13,20–25]. In this section, some of these inorganic nanomaterials are described in detail.

3.2.2.1 Quantum Dots

Quantum dots are semiconductor nanomaterials in the size range of 1–10 nm, which has the ability to produce fluorescence when excited by any light source like a laser. In the quantum dots, the crystalline core is present, and based on its composition and size, the fluorescence will be produced [79]. The composition of the core can consist of cadmium–selenium, cadmium–tellurium, indium–phosphate, and others. The shell is made up of zinc–sulfide to stabilize the core of the molecule. The shell also improves the optical and physical properties of the quantum dots along with

enhanced bioavailability and biocompatibility of the material. To improve the water solubility and reactivity of the material, these structures are additionally capped or encapsulated with other materials such as PEG and silica [80].

Quantum dots are advantageous nanomaterials in terms of their various properties like physical stability, higher signal to noise ratio, greater photostability than other dyes, and higher resistance to degradation, which help in tracking cells for a longer period. Moreover, these quantum dots exhibit broader excitation and narrow emission spectra, so one single light source can excite multicolor quantum dots without overlapping, and show 10–20 times brighter than organic dyes. Additionally, the longer fluorescent lifetime and flexibility to get molded into different shapes make quantum dots a good contrasting agent for imaging.

Quantum dots are used in various biomedical applications including encapsulation of hydrophobic drugs with other functionalities for targeting the diseased site, tags for organic and inorganic drug carriers, and a molecular imaging agent for fluorescent microscopy. These can be utilized in magnetic resonance imaging (MRI) as a molecule that can detect apoptosis when linked with MRI agent, i.e. gadolinium. Quantum dots are also studied as a non-invasive optical molecular imaging agent to early detect, diagnose, and monitor the treatment of cancer, as a locator or marker of tumor cells, and for identification of the target DNA sequence in gene technology. These can also act as a fluorophore for pathogen and toxin detection, and as an agent that can detect, visualize, measure, and track the molecular events in the field of neuroscience [81,82].

Hani NA et al. used cadmium sulfide quantum dots technology in their research to load sesamol, a cytotoxic agent. These were modified with chitosan in order to improve its anticancer activity. For characterization of these prepared quantum dots, X-ray diffraction, thermogravimetric analysis, UV-Vis spectroscopy, zeta potential, electron microscopy, Fourier transform infrared spectroscopy, electron diffraction and fluorescence emission techniques were used. The cytotoxic assay of cadmium sulfide with chitosan, sesamol (free drug), and chitosan-modified sesamol-loaded cadmium sulfide quantum dots showed the half-maximal inhibitory concentrations values as 1730 ± 54 , 495 ± 16.4 , 117 ± 3.2 $\mu\text{g/ml}$, respectively. The cadmium sulfide quantum dots modified with chitosan were found to be more active against tumor tissues compared to the free drug [83].

Graphene quantum dots (GQDs) are zero-dimensional nanomaterials and have decent biocompatibility with low toxicity profile. In one controlled photodynamic therapy, GQDs were used as a photosensitizer to regulate the generation of reactive oxygen species by varying the doping amount of nitrogen atoms. Further, for charge reversal, (3-aminopropyl) triethoxysilane (APTES) had been conjugated on the surface of GQD to target nucleus. The results demonstrated that prepared GQDs had a dual role, i.e., nucleus-targeted drug delivery and photosensitizer. Thus, this can be a novel approach for multifunctional therapy using quantum dots [84].

3.2.2.2 Gold Nanoparticles

Gold nanoparticles (AuNPs) are small gold particles in the size range of 10–100 nm used for the delivery of drug molecules. Gold has been studied in biomedical

research for many years. Moreover, AuNPs have gained focus in biomedical applications because of their unique properties and surface characteristics [85]. Because of the versatility of surface chemistry of AuNPs, it can be conjugated or coated with small molecules, polymers, antibodies, oligonucleotides, proteins, and biological recognition molecules.

AuNPs are spherical molecules with high surface-to-volume ratio, great biocompatibility, and low toxic potential [86]. The color of AuNPs varies in the range from brown, orange, red, to purple colors in the aqueous phase which depends on the size of the core. Based on its color variation and absorption spectra, these nanoparticles have a property of surface plasmon resonance. Due to the different kinds of properties, AuNPs have applications in various fields including electronic devices, electrochemical sensing, imaging, photothermal therapy, sensory probe, drug and gene delivery, and contrast agent for diagnosis and phototherapy.

To deliver or improve the delivery process of therapeutics to cells, AuNPs are used for passive and active targeting. For passive targeting, the EPR effect is exploited, while for active targeting, various surface-modified ligands have been confiscated which targets explicitly to the desired cells or tissues. In these nanoparticles, drugs can be loaded either covalently or by non-covalent interactions [87]. The surface modification of AuNPs with cellular targeting improves the circulation characteristics, minimizes the aggregation rate, and improves the targeting of therapeutic [88].

The concept of AuNPs has been utilized to bypass the BBB by targeting insulin receptors. Insulin receptors are targets for the treatment of various neurodegenerative disorders. In one study, insulin-coated AuNPs were prepared and analyzed for its ability to cross BBB to deliver the drug into the brain. The *in vivo* examination revealed the wider distribution and accumulation of 20 nm-sized insulin-coated AuNPs within the brain after 2 hours of injection. The CT imaging exposed the fact that these particles were traveled to the desired insulin receptor-rich region of the brain. This observation supports the potential use of AuNPs for the delivery of therapeutic agents across BBB to targeted sites [89].

Nihal et al. developed multifunctional magnetic gold nanoparticles (MGNPs) to deliver the drug selectively to the tumor site and to encourage photothermal therapy by the production of heat via near-infrared laser absorption. Further, these nanoparticles were coated with PEG and conjugated with DOX. The prepared theranostic nanoparticles showed great efficacy of chemo-photothermal therapy and enhanced cancer management by reducing the damage of normal tissue surrounding tumor cells. Thus, these can be a novel platform technology for cancer treatment with multiple applications [90].

3.2.2.3 Magnetic Nanoparticles

Magnetic nanoparticles are a core-shell type of geometry that is made up of magnetic or metallic material with the encapsulation of polymer or metallic coating. Therapeutic agents can be attached to these nanoparticles by functionalizing the coating metal or polymer. These drug-complexed nanoparticles are then injected

into the bloodstream near the target site, and the magnetic fields are concentrated at the target sites [91].

Chemical properties, size, and magnetic properties of magnetic nanoparticles play an important role in the drug release behavior and the therapeutic action of the nanoparticles. The surface chemistry of the nanoparticles is helpful in the protection of the system from the reticuloendothelial system (RES) in order to increase the half-life of the drug in the blood. For drug loading, two strategies have been exploited, namely, chemical linkages between ligand molecules on the magnetic nanoparticle surface, and physical interactions like electrostatic, hydrophilic/hydrophobic, and affinity interactions. To stabilize magnetic nanoparticles, amphiphilic comblike polymers such as comblike PEG derivatives can be considered helpful. Both passive and active targeting is possible with these molecules. Furthermore, controlled drug release can be achieved using static or alternating magnetic fields [92].

Due to the above-mentioned advantages, the magnetic nanoparticles have gained the potential to treat and diagnose several diseases such as cancer, musculoskeletal system disorders, chronic kidney diseases [93–95].

In a study, DOX-loaded superparamagnetic iron oxide nanoparticles (SPIONs) were prepared and modified using a heparin–Poloxomer (HP) shell for targeted drug delivery. The entrapment efficacy was found to be $66.9\% \pm 2.7\%$, and the preparation exhibited a controlled release for about 120 hours. The *in vitro* assay of the prepared nanoparticles revealed that DOX-loaded SPION-HP could exert more significant anticancer effect against HeLa cells. Therefore, these can be safely used for targeted drug delivery in cancer treatment [96].

Magnetic nanoparticles are used for their property of burst release followed by controlled release. In one study, magnetic nanoparticles for hydrophobic drugs had been formulated by the coating of Fe_3O_4 nanoparticles with polydopamine, which was further functionalized with 6-thio- β -cyclodextrin (6-thio- β -CD). Diclofenac was used as a model drug to inspect the interaction of prepared magnetic nanoparticles with hydrophobic drugs. The results exposed that the prepared nanoparticles were able to load higher drug concentrations and showed burst release followed by a sustained release profile. Therefore, this study confirmed the potential of magnetic nanoparticles as a drug delivery vehicle [97].

3.2.2.4 Plasmonic Nanoparticles

Plasmonic nanoparticles are noble metal nanostructures that have the ability to manipulate light at nanoscales and generate heat [98]. For the preparation of these nanoparticles, plasmonic materials such as metals or metal-like materials having negative real permittivity are used. The most representative examples of plasmonic materials are gold and Ag. Other plasmonic materials exhibit optical properties like metals at a specific wavelength. So, plasmonic nanoparticles prepared from plasmonic materials which can absorb light of specific wavelength can be used for biomedical applications [99]. These metal nanoparticles have tunable optical properties by changing the characteristics of size, shape, and composition. Owing to this property, plasmonic nanoparticles generate heat by converting

absorbed light into heat. These nanoparticles are used in various delivery strategies extending from photothermal to trigger drug delivery systems. The unique property of localized surface plasmon resonance (LSPR) distinguishes plasmonic nanoparticles from other inorganic nanoparticles [100].

For drug delivery applications, the therapeutic moiety is directly linked to plasmonic nanoparticles through a thermo-sensitive linker [101]. When light is irradiated on nanoparticles, the heat is generated and resultantly breaks the linker and ultimately releases the drug. Other than drug delivery purposes, these nanoparticles are also used for imaging and diagnosis of diseases. Additionally, these are also used for the photothermal demolition of cancer cells or toxic aggregates of proteins. Plasmonic nanoparticles are being examined for their exercise in controlled drug delivery systems. For biomedicines, these particles act as light-activated nanoscopic heaters and are specially used for photothermolysis of cancer cells [102]. Other than this, noble metal nanoparticles are used for selective targeting and molecular imaging, destruction of bacteria or viruses, controlling the specific localized breakdown of proteins and nucleic acids, and therapeutic purposes [103]. Surface modification of nanoparticles helps in targeting therapeutics to desired biological systems in the body.

An *in vivo* study had been performed for evaluation of enhanced drug-carrying efficacy and cardiac anti-hypertrophy potential of nano-curcumin-encapsulated photo-plasmonic nanoparticles. For this study, curcumin-capped gold-loaded PLGA nanoparticles (CAu-PLGA nanoparticles) were made utilizing a double-emulsion solvent evaporation method. This work presented the application of CAu-PLGA nanoparticles in the prevention of hypertrophy in the Wister rat model. Enalapril was used to induce the hypertrophy in the rat model. The *in vivo* study results demonstrated that the designed CAu-PLGA nanoparticles significantly exhibited an anti-hypertrophic effect compared to other groups. On further analysis, they found that CAu-PLGA nanoparticles increased the survival rates in the rat model and also improved the cardiac functions including heart weight, systolic, and diastolic functions. These effects were linked to the therapeutic effects of the formulation which resulted in increased delivery of drugs and inhibited cholesterol accumulation, which ultimately prevents myocardial infarction [104].

3.2.2.5 Halloysite Nanoclay

Halloysite is a naturally occurring material obtained from weathered rocks and soils in tropical and subtropical areas, especially from volcanic ash. It is a naturally abundant and viable aluminum silicate clay mineral with the empirical formula $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})4\text{nH}_2\text{O}$ [105]. In terms of morphology, they can be found in different structures like tubular, platy clays, spheroidal, and other. The most common form of halloysite is halloysite nanotubes (HNTs), which is a hollow tubular nanomaterial that is chemically the same as kaolin. Its dimensions include 500–1500 nm length with 10–20 nm inner and 40–70 nm outer diameters. Based on the functional groups present in the inner and outer sides, HNTs are positively charged from the inner side and negatively charged from the outer side. This allows the encapsulation of negatively charged molecules like DNA inside

the core at a lower pH [106]. The charge on HNTs provides the opportunity for several surface modifications. These nanomaterials are considered to be a potential candidate as a drug carrier because of its properties of non-toxic, natural origin, biocompatibility, good dispersion behavior, fine particle size, and larger surface area. It holds a great mechanical strength which helps protect the negatively charged encapsulated drug from the outside environment [107]. It also has large cation exchange property which makes it a suitable option for the delivery of both hydrophilic and hydrophobic molecules. Other than this, HNTs possess some additional properties like prolonged drug release time, longer duration of action, reduced frequency of administration, higher drug loading, regeneration ability, high porosity, antibacterial, and anti-inflammatory activity [108], which make them a distinguishable nanomaterial from other systems.

Because of its unique behavior, HNTs have shown its applicability in different fields, including drug delivery, gene delivery, tissue engineering, wound healing, cancer therapy and isolation of stem cells, and biosensing in many different fields. By modification of the characteristic of HNTs, other benefits can also be achieved. One such study had been conducted in which the sustained release behavior of sodium salicylate using modified halloysite was analyzed. For this study, composite polymer–halloysite modification and acid treatment were done and after that drug was loaded and analyzed. Here, the formulation was evaluated using *in vivo* studies to compare its release behavior with unmodified HNTs. The consequences from different characterizations for modified HNT formulation had shown that acid treatment and composite formulation did not affect the tubular structure; instead, the acid treatment modification improved the loading of sodium salicylate. This improved loading resulted in the sustained release behavior of the larger amount of the drug from the formulation. The polymer halloysite composite resulted in delayed drug release compared to acid-treated and untreated HNTs, which might be because of the formation of a consistent layer of polymer around halloysite during formulation. Thus, modified halloysites can provide sustained drug release profiles [109].

Liu et al. studied HNTs for the delivery of RIPK4 small interfering RNA for the treatment of bladder cancer. RNA interference technology is considerably used for silencing the expression of a particular gene, so these researchers used the HNT approach for the delivery of small interfering RNA. From the study results, it had been confirmed that when HNT/siRNA complex was used, it enhanced the serum stability of siRNA, which in turn increased the circulation time of the molecule in the blood and encouraged the tumor cell uptake and accumulation. On analysis of the activity of siRNA, it had been reported that HNT/siRNA silenced the expression of the RIPK4 gene more efficiently in bladder cells and resulted in the downregulation of bladder tumors with no adverse effects [110].

3.2.3 COMBINED ORGANIC AND INORGANIC NANOMATERIALS

When the advantageous characteristics of organic and inorganic nanoparticles are combined, the organic–inorganic hybrid can be obtained, and this can be

done by modification of inorganic nanoparticles with organic materials or by the application of organic species as an outline for the controlled growth of inorganic materials [8]. Figure 3.1 illustrates the schematic representation of the application of organic, inorganic, and hybrid nanomedicines in the biomedicine field. Several interactions are involved in the development of hybrid nanoparticles. The development of these nanoparticles provides an opportunity to merge the properties of both organic and inorganic nanomaterials for drug delivery purposes. Organic materials used in these nanomaterials are generally polymeric materials that are easily modifiable, while inorganic materials used is tunable at the atomic level. When the metal present in the center interacts with heteroatomic organic ligands, polarized nano-salt structures are generated, which are completely different from non-hybrid structures [111].

Many hybrid nanostructures have been developed by scientists as an application in many different fields. In the pharmaceutical formulation research, these particles have exploited the beneficial feature of organic and inorganic materials. These resulted in the drug's release profile, based on the permeation of water from the outer shell and erosion and degradation of inner core material by the process of hydrolysis. For the formation of hybrid nanoparticles in the core portion, different polymers, lipids (including cationic, anionic, zwitterion, and neutral phospholipids), and inorganic materials (like iron oxide, silica, etc.) are incorporated. In contrast, organic materials like polysaccharides are used to encapsulate active pharmaceutical moiety in the outer shell.

The lipid part acts as an intermediate layer over inorganic/polymeric core, improves the biocompatibility of the system, behaves like a barrier for drug leakage, and controls the water permeation. The outer organic layer is a functional layer having specific ligands that are used to target or increase circulation and retention time. To this layer, different antibodies or aptamers or other moieties can also be connected by modification of the outer layer with suitable charge. Different methods could be employed to develop different kinds of hybrid nanoparticles.

With these hybrid nanoparticles, targeted and controlled drug delivery with effective drug-carrying capacity can be achieved. Some factors such as lipid-polymer ratio, PEGylation, and polymer nature can influence the physicochemical properties of hybrid nanoparticles. When there is a lower lipid-polymer ration, there are chances that particles are not coated with lipids, which can result in bridge formation of lipids with other particles and initiating aggregation of particles. While at a higher ratio, decreased production yield may be obtained because when there is a large lipid amount, the whole amount cannot be incorporated, and the remaining free lipids can form liposomes and thus affect the homogeneity of the formulation. So, the optimized ratio of lipid-polymer should be there to obtain functional hybrid nanoparticles. PEGylation can prolong the circulation time by inhibiting the aggregation and opsonization of particles. The chain length of coated PEG-lipid and the molar ratio of PEG-lipid affect the colloidal stability of the formulation. The nature of the polymer includes the size and surface charge characteristics of the polymer. The density of the polymer affects the stability

and particle size, while surface charge influences the adsorption of lipid over the polymeric particles [112,113].

In many different fields, hybrid nanoparticles have gained interest. In a study, the controlled release behavior, improved selectivity, and efficiency of the drug were obtained using hybrid nanoparticles. For this purpose, iron oxide nanoparticles had been prepared, and further, the surface was modified by diazonium chemistry and coated using chitosan and chitosan-grafted polylactic acid. DOX was the model drug that has been used in the study. The evaluation results confirmed that the particles were spherical with coating. These nanoparticles were found to have a greater stability profile along with increased encapsulation efficacy for the drug. The enhanced cytotoxic behavior of DOX had been demonstrated when loaded in hybrid nanoparticles, which proved the benefits of the prepared formulation over simple iron oxide nanoparticles [114].

3.3 CONCLUSION

The pathological conditions in diseased tissues exhibit a great impact on the effective delivery of drugs. Compared to conventional drug delivery systems, the nanotechnology-based drug delivery systems present several benefits like the controlled release of the drugs, dose reduction, targeted site-specific delivery of therapeutics to specific areas in the body, and combined diagnostic tools as theranostic agents. Thus, nanomedicines make the treatment cost-effective, non-invasive, and non-toxic by diminishing the side effects of the therapeutic dose of drugs. But, the efficacy of nanoparticles as delivery vehicles depends on the size, shape, and physicochemical characteristics. Currently, the nanomedicine of organic and inorganic materials has been extensively explored for biomedical applications. The organic nanomedicines such as liposomes are biocompatible because these mimic the physicochemical conditions experienced in biological tissues. Therefore, organic nanomedicines have been used in imaging and therapeutic agents. On the other hand, the inorganic nanomedicines offer great promise in diagnostics, biosensing, and drug delivery. Further, the combination of both organic and inorganic materials into one single system as a hybrid nanomedicine not only exhibits advantages of these two types but also possess the ability to systematically tune hybrid materials. Thus, these are promising nanomedicines for biomedical applications.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

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4 PLGA-Based Nanoparticulate Systems

New Trends in Nanomedicine

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4.1 INTRODUCTION

The application of nanotechnology in drug delivery has greatly influenced its scope and further strengthened its stand as a suitable alternative to conventional treatment. In this perspective, a large volume of research has been focusing on discoveries of new methods and drugs of the treatment of a range of diseases, wherein the emphasis is on the disease itself along with controlling drug-associated side effects. In this context, the drug targeting to specific organs by avoiding other nonspecific tissues has been actively pursued for decades, and effective drug delivery is one such method that has been revealed its efficiency and safety. By controlling the amount of drug dose for its optimum performance, the efficiency of the drug can be further enhanced. This can be achieved with the help of nanotechnology concepts because nanotechnology may offer (i) improvised transport of water-insoluble drugs, (ii) increased specificity towards the cell of interest, (iii) transport of drugs across junctions such as blood–brain barrier (BBB) that in normal circumstances would not allow the movement of drugs, (iv) enormous potential in quantum properties, and (v) large surface to mass ratio, which can facilitate as a carrier for compounds having either therapeutic or diagnostic values (Farokhzad et al., 2009; De Jong et al., 2008).

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer that has undergone intense research and is regarded as one of the best-defined polymers for drug delivery (Figure 4.1a). Polymeric nanoparticles (NPs) typically range from 200 to 400nm, whereby the drug of interest is either absorbed or encapsulated into the polymer matrix or adsorbed or conjugated onto its surface (Alimohammadi et al., 2014) (Figure 4.1b). Its biodegradability characteristics have made it suitable for human use and being among the few polymers approved by the Food and Drug Administration (FDA), USA; its resourceful properties have been exploited and experimented on a wide range of diseases.

PLGA has found its way into many clinical studies for development of nano-carriers in various biomedical applications notably, vaccination, bone healing, and infections among other diseases.

The properties, *viz.*, low toxicity, compatibility with biological materials such as tissue and cells, and ability to control the release of drug from PLGA, have made it suitable for a targeting multiple organ in humans.

It can be easily formed into drug carriers of any desired scale, from millimeter-sized implants to fine coatings of medical instruments; from microparticle to NP drug carriers while encapsulating drugs of different molecular characteristics and sizes (Semete et al., 2010). PLGA nanomaterials are synthesized through the random ring opening co-polymerization of the cyclic dimers (1,4-dioxane-2,5-diones)

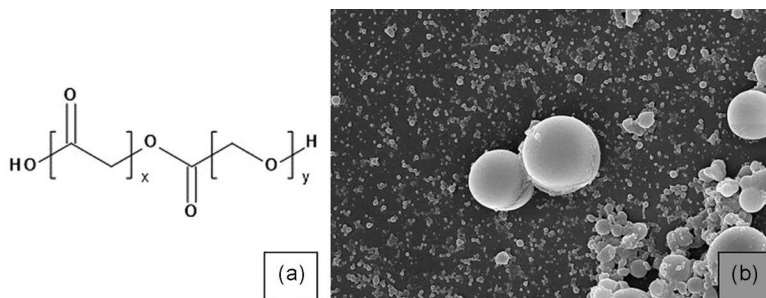


FIGURE 4.1 (a) Chemical structure of poly(D,L-lactic-co-glycolic acid) where x represents the number of units of lactic acid and y represents the number of units of glycolic acid. (b) SEM image of PLGA nanoparticles.

of the two key ingredients: lactic acid and glycolic acid. These monomers being endogenous in nature are easily metabolized through the Krebs cycle, accompanied with a negligible systemic toxicity when used for drug delivery or other biomedical systems (Shi et al., 2018). In recent studies, it was reported that the oral administration of PLGA nanomaterials in an animal model (rat) exhibited no toxicity in important vital organs, i.e., brain, kidney, and lung, whereas the minimal toxicities observed on the liver and intestine suggest the utilization of PLGA NPs for the delivery of oral drugs (Navarro et al., 2016).

Many factors are considered when PLGA NPs are fabricated as a drug vehicle. Biodistribution, optimal concentration, and time duration for release are some of the main factors that are prominent, and altering these factors can tailor NPs to develop certain specific characteristics. Therefore, design specifics such as material, geometry, and location also integrate release mechanisms, degradation properties, and clearance of the vehicle as well as active pharmaceutical ingredients (APIs). Biodistribution profile revealed that fluorescently labeled PLGA NPs were most identified in the liver at $40.04\% \pm 8.42\%$, trailed by the kidney at $25.97\% \pm 7.09\%$, and the heart at $11.92\% \pm 3.16\%$, and the least concentration was in the brain ($12.86\% \pm 2.82\%$) for up to 7 days of experimentation (Horvati et al., 2018). As reported earlier, drug release behavior of PLGA NPs includes either (i) its transportation through pores filled with water, (ii) transport directly through the polymer, or (iii) transport through dissolution of the polymer. In most circumstances, an initial burst of drug release is observed followed by a steady release of drug which is then cleared by the respiration in the lung. Several factors affect the drug release profile such as the type of polymer, L:G ratio, drug characteristics, location of target organ, and additives. Due to the complex nature of drug release profile, it is difficult to predict the nature of release mechanism. As a result, many mathematical models have been developed which give a rapid general view and basic understanding into the governing release mechanism or the methods inducing the drug release.

4.2 PREPARATION OF PLGA-BASED NPs

1. **Emulsification-evaporation method:** It is considered as the simplest, quickest, and commonly used procedure for the synthesis of PLGA NPs. Nanomaterials can be subdivided into single (oil in water [o-w]) emulsion, double (water in oil in water [w-o-w]) emulsion, multiple (single, double, or multiple) emulsion, and polydispersed systems where both [o-w] and [w-o] emulsions exist concurrently and stabilized by the addition of lipophilic or hydrophilic surfactants. When hydrophobic drugs need to be encapsulated, the [o-w] emulsion process is employed wherein the drugs along with PLGA need to be dissolved in organic solvents such as chloroform or dichloromethane that are immiscible after which they are further homogenized in the second aqueous phase that contains another surfactant such as poly(vinyl alcohol) (PVA). The homogenization process can be achieved using a homogenizer or ultrasonic processor. In the last stage of the process, the organic solvent is evaporated and the PLGA NPs can be obtained by downstream processing including centrifugation, washing, and lyophilization (Swider et al., 2018; Mir et al., 2017). For encapsulation of hydrophilic drugs, the w-o emulsion with double or multiple emulsion method is generally preferred. Under the influence of vigorous stirring, drugs dissolved in an aqueous solution are added to PLGA dissolved in organic phase to obtain [w-o] emulsion. This emulsion is further added to another aqueous solution that contains a surfactant under the influence of homogenization or ultrasonication to form a [w-o-w] emulsion. Then, the hardened NPs are retrieved through the evaporation of solvent (Rezvantabolab et al., 2018). By adjusting parameters such as stirring speed and input rate, we can effectively encapsulate hydrophobic and hydrophilic drugs in NPs of nano or micron sizes (Allahyari et al., 2016). The disadvantages of this method are the need for heat or a vacuum to evaporate the solvent and the incomplete removal of stabilizer (Rezvantabolab et al., 2018).
2. **Nanoprecipitation method:** This method is based on the principle of precipitation wherein the addition of a non-solvent to the polymer solution results in the precipitation of the polymer. This occurs in a series of steps, i.e., super saturation, nucleation, growth by condensation, and coagulation leading to the creation of polymer NPs. In this method, PLGA and hydrophobic drugs are dissolved in polar organic solvents, i.e., dimethyl sulfoxide (DMSO), acetone, ethanol, etc. and injected at a controlled speed into an aqueous solution containing surfactant. This will allow for the formation of PLGA NPs by the rapid diffusion, and NPs can be collected after the solvent elimination (Dinarvand et al., 2011). Nanoprecipitation method is used to load hydrophobic drugs into the <100 nm particle size (Swider et al., 2018). This method can easily be scaled up, requires low energy, and have high reproducibility. However, this method has low encapsulation efficiency for hydrophilic drugs and has big particle size variation (Rezvantabolab et al., 2018).

3. **Spray-drying method:** This is a relatively easy, quick, reproducible, and easy to upscale technology which can be used for encapsulation of heat-sensitive biopharmaceutical compounds containing both hydrophilic and hydrophobic drugs. Briefly, hydrophilic drugs dissolved in an aqueous solution is subjected to vigorous stirring with an organic phase in which PLGA is dissolved. Through the vigorous stirring, [w-o] emulsion is formed. Further, the PLGA NPs are produced by spraying method or by using a stream of heated air (Booyesen et al., 2013). The disadvantage of such process is low productivity of PLGA NPs (Ding et al., 2018).
4. **Microfluidics method:** Microfluidics is a device that contains micro-channels in which micro- or nanoliters of liquids are operated in a controlled manner for various parameters, i.e., mixing, heat, and/or mass transport. Microfluidics methods are generally categorized into two main categories: droplet-based (segmented) and continuous microfluidics. The continuous-phase flow microfluidic systems are used to synthesize PLGA NPs by formation of single emulsions (Swider et al., 2018). The microfluidic methods are superior and result in the surface-functionalized PLGA NPs with a narrow size distribution, possess high reproducibility, and also prevent the initial burst release of the drug (Rezvantlab et al., 2018). The main limitations of this method are microchannel clogging and fouling, and scale-up is very difficult (Rezvantlab et al., 2018).

4.3 PHYSIOCHEMICAL PROPERTIES OF PLGA NPs

1. It is amalgamated by copolymerization of glycolic and lactic acids. Glycolic acid is a translucent hydrophilic polymer with less water soluble and higher degradation rate under specific physiological conditions, whereas lactic acid is a rigid and hydrophobic polymer with a minimal mechanical strength. PLGA inherits the intrinsic properties of both the biomolecules and as a result the lactic acid/glycolic acid ratio, and molecular weight of the polymer robustly affects its rate of degeneration. For example, with an increase in the LA/GA ratio, the hydrophobicity of PLGA also increases, which results in lower degradation and slower drug release rate. In addition, the final molecular weight also affects the degradation process and drug release profile of NPs, i.e., as the molecular weight decreases, there is an increase in both degradation and rate of drug release. Critical factors such as degradation, release kinetics, and molecular weight directly affect the size of the synthesized NPs and influence the therapeutic performance of PLGA NPs. Though formulations that are larger in size have a higher drug loading capacity, producing lower size range (nano/micro) formulations is fundamentally essential for the ability of NPs to cross biological barriers and travel to the intended location (Rezvantlab et al., 2018).
2. **Solubility of PLGA:** PLGA containing less than 50% glycolyl units can be dissolved in most organic solvents, i.e., halogenated hydrocarbons

(chloroform and dichloromethane), ethyl acetate, dioxan, tetrahydrofuran, and acetone. Conversely, PLGA having 50% and a higher number of glycolyl units is insoluble in most organic solvents, as well as in solvents like hexafluoroisopropanol; hence, it is involved in the characterization process of PLGAs that have a high number of glycolyl units (Avgoustakis, 2008).

3. **Crystallinity:** The chemical and stereo isomeric compositions of lactide units in the copolymer, i.e., 0%–75% glycolyl units in poly(D,L-lactide-co-glycolide) and 25%–75% glycolyl units in poly(L-lactide-co-glycolide), both are amorphous (Avgoustakis, 2008).
4. **Thermal stability of PLGA:** Copolymers in PLGA are thermoplastic in nature, i.e., they exhibit heat stability when it is not subjected to moisture (Avgoustakis, 2008). When the temperature dips, thermal degradation can be estimated as a function of time and temperature and is substantially enhanced by contaminations, residual monomers, and humidity.

4.4 FABRICATION OF PLGA-BASED NANOCARRIERS AND THEIR FUNCTIONALIZATION

Several categorizes exist to group PLGA-based NPs, which highly depends on the process of preparation and difference in the structural organization. Drug encapsulation can follow one of the two scenarios, wherein the drug is either entrapped inside the core of the NP or it gets adsorbed on the surface of the nanosphere (Shi et al., 2018). The method used for preparation of PLGA NPs is based on the specific requirements for transport and the chemical property of the compound to be transported. Hence, many techniques have been developed over the years for the synthesis of PLGA NPs (Astete and Sabliov, 2006). The preparations from the polymerization of monomers include the bottom-up approaches such as emulsion, mini-emulsion, micro-emulsion, and interfacial polymerization. The top-down approach involves techniques such as supercritical fluid technology, solvent evaporation, nanoprecipitation, salting out, dialysis, and emulsification/solvent diffusion. Water-in-Oil-in-Water (w1/o/w2) emulsion solvent evaporation process is used when encapsulating hydrophilic drugs in PLGA NPs, while Oil-in-Water (o/w) emulsion solvent evaporation is used when encapsulating hydrophobic drugs. During the synthesis process, many factors directly or indirectly contribute to the final size and polydispersity of NPs. For example, surfactant concentration, type of surfactant, concentration of PLGA in the organic phase, the volume ratio of aqueous to organic phase, organic solvent used, and sonication time and power all contribute to the final particle size (Ahmad et al., 2018).

As demonstrated in a recent work, the lack of cell recognition sites has restricted the application of PLGA in many areas of medical science. Yuan and coworkers successfully modified porous PLGA microspheres with poly-L-lysine (PLL) which could facilitate cell growth on the microspheres (Upadhyay et al., 2014). Such modification has the prospective applications in the field of tissue engineering (Singh et al., 2017). The modification made the NPs bacteriostatic

against pathogens such as *Escherichia coli* in addition to increasing the effectiveness of tetracycline against the pathogens. Additionally, NPs were found not to exhibit cytotoxic effects against fibroblasts. Mucoadhesive properties of nano-systems greatly hinder the transport of NP drugs by preventing them from crossing the mucosal sites. This issue can be addressed by modifying the surface of PLGA NPs with linear, low-molecular-weight polyethylene glycol (PEG) has made drastic advancements in the distribution of drug conjugates at the mucosal sites (Ali et al., 2013). To increase specificity to target cells, PLGA NPs have undergone various forms of surface modification. Surface modification has been a key factor that can be exploited for drug delivery to any body organ. PLGA NPs surface modified with chitosan for oral delivery of tolbutamide have shown chitosan chains to improve the thermostability of the original PLGA NPs (Nance et al., 2012). A nonionic, surfactant Pluronic F127 has been reported to prevent aggregation of PLGA NPs developed as a carrier of Tuftsin, for the treatment of tuberculosis (Olivier, 2005). Other instances include third-generation P-glycoprotein GF120918 for effective oral delivery of Docetaxel, and a cytotoxic taxane was established (Dai et al., 2018). Numerous APIs have been encapsulated in PLGA NPs that have proven to have a strong therapeutic potential when compared to drug alone in both *in vivo* and *in vitro* studies.

The process of “manipulation of fluids in channels with dimensions of tens of micrometers” where the fluid behavior can be controlled is termed as “microfluidics.” In this procedure, a continuous flow system ensures excellent uniformity in NPs synthesized which is otherwise absent in conventional methods. Such devices can quickly evaluate the interactions between NPs and biological systems by mimicking the important aspects of the *in vitro* and *in vivo* systems. First proposed by Thorsen et al. in 2001, microfluidics has been studied for its use in various applications (Tetsuya et al., 2005). Martins and coworkers encapsulated anti-HIV drug Efavirenz in PLGA NPs using microfluidics platform for drug delivery to the brain. The results suggested that when prepared through the conventional method, NPs produced through microfluidics presented reduced size, good polydispersity, and higher drug loading efficiency (Arya et al., 2018).

PLGA NPs have extensive applications in medicine (Figure 4.2). Being endogenous to human body, their monomers can be easily metabolized through the Krebs cycle. As a result, systemic toxicity associated with PLGA is very low. Its diverse applications can also be attributed to its ability to change its physiochemical properties, drug release rate and behavior during transport by manipulating the mode of synthesis, molecular weight, or the attachment of special ligand with regard to the diseases in study (Park et al., 2017). Their biocompatibility and biodegradability properties have been exploited in numerous studies for a wide range of medical disorders. Several disease-related drugs have been successfully encapsulated in PLGA NPs. Such drug formulations have superior advantages over conventional medicines in aspects such as better control of drug release, targeted delivery, and therapeutic effect. The applications of PLGA NPs in the most important therapeutics have been briefly discussed below.

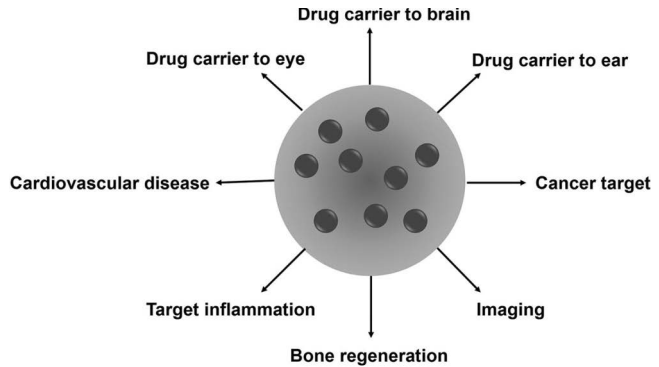


FIGURE 4.2 Broad application of PLGA nanoparticles in medical industry.

4.5 PLGA NPs FOR DRUG DELIVERY TO THE BRAIN

The complexity surrounding drug targeting to the brain tissues is intense due to the existence of the BBB. The BBB presents itself as an integral part of the nervous system. As a part of defense mechanism, it physically restricts the access of biomolecules such as carbohydrates, peptides, and proteins which pass *via* the brain capillary endothelial cells. As a result, many therapeutic drugs, antibiotics, and vaccines are inhibited (Zhao et al., 2017) (Figure 4.3). The tight junctions located between endothelial cells, loss of fenestrae, and lack of macropinocytosis are the main reasons for the barrier function of the BBB (Mieszawska et al., 2012). A PLGA NP across a human placental was shown to be a success (Chen et al., 2009). *Ex vivo* studies revealed that PEG-coated PLGA NPs could successfully breach the BBB and quickly enter the rat brain tissue, when compared to control study (Mariano et al., 2014). In such delivery system, combinations

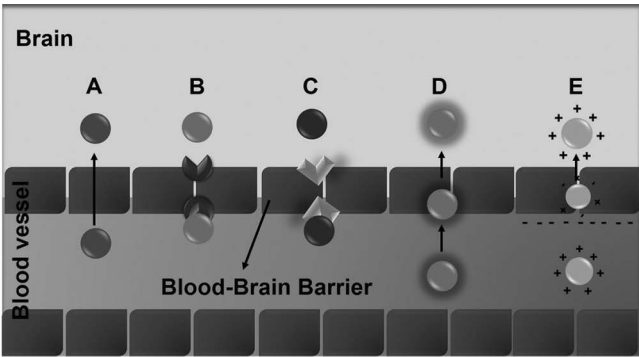


FIGURE 4.3 Schematic representation of the mechanisms of passage of substances across the blood–brain barrier. (a) Passive diffusion of lipid-soluble molecules, (b) carrier-mediated transport (CMT), (c) receptor-mediated transcytosis (RMT), (d) adsorptive-mediated transcytosis (AMT), and (e) cell-mediated transcytosis (Singh et al., 2017).

of hydrophobic PLGA with surface coating with a hydrophilic PEG group were able to avoid opsonization and phagocytosis with enhanced release kinetics in the blood (Fornaguera et al., 2016).

PLGA NPs can be further engineered for carrying therapeutic agents specifically to the brain. For instance, Kaur and coworkers encapsulated analogues of thyrotropin-releasing hormone in PLGA NPs which were further coated with chitosan (Dorati et al., 2017). Chitosan possess mucoadhesive properties that are exploited for effective intranasal delivery of drugs to the brain. The transports of NPs to the brain by intranasal administration were proven to be successful and may deliver an effective mode of delivery of drugs to the brain for the treatment of multiple neuro-related ailments.

4.6 PLGA NPs FOR DRUG DELIVERY TO THE EAR

Anatomically, the inner ear is barricaded within the otic capsule and is completely isolated from its surrounding organs. The thickness of the otic capsules prevents drug diffusion to occur. Currently, the treatment for disorders related to the inner ear involves the use of systemic delivery, intratympanic injection, or surgery with each having its own drawbacks. Though systemic delivery is safe and non-invasive, it lacks specificity accompanied with subtherapeutic concentrations. On the other hand, intratympanic injection involving the filling of the inner ear with drugs results in drug clearance from the eustachian tube and/or irregular diffusion rate and the possibility of damage to the inner ear structure (Betsy Szeto MPH). Cochleostomy which involves the direct injection of drugs to the ear increases the specificity compared to the other methods but is invasive and increases the chance of trauma and infection (Betsy Szeto MPH). The anatomic barriers associated with the inner ear prevent the delivery of drugs into the ear. The blood-labyrinth barrier (BLB) effects systemic delivery, while the round window membrane (RWM) prevents intratympanic injection. The RWM plays the role of the primary barrier for delivery of therapeutic agents to the inner ear via local application. A recent study had reported the transport mechanism of PLGA NPs across the RWM. The study revealed that PLGA NPs penetrated across RWM via the transcellular pathway. NPs approached the epithelium barrier; it was observed that micropinocytosis was the main path for the uptake of PLGA NPs. PLGA NPs containing interferon α -2b was encapsulated in chitosan/glycerophosphate (CS/GP)-based thermosensitive hydrogel. This was set up for the delivery of IFN by intratympanic injection (Dorati et al., 2017). This system allowed lengthier delivery time for the transport of protein drugs to the inner ear, which could inevitably become a successful vehicle for the transport of drugs to the inner ear. Similarly, Tamura and coworkers examined the efficacy of loading Rhodamine in PLGA NPs for drug delivery to the cochlea (Zhang et al., 2018b). It was noticed that when the NPs were placed on the RWM, the NPs were also found on the Scala tympani, demonstrating the ability to the nano-formulation to permeate through the RMW. In this study, the NPs showed excellent targeted delivery ability to the cochlea when compared to systemic application.

4.7 PLGA NPs FOR DRUG DELIVERY IN CANCER TREATMENT

One of the leading causes of deaths worldwide, the demand for an effective therapy in cancer treatment continues to rise. The lack of specificity in conventional modes such as chemotherapy has resulted in search for alternative approaches. The use of NPs as drug delivery tools has been extensively researched owing to their advantages such as the ability to deliver drugs to specific tumor targets at precise dose, thus enhancing the overall effect of the drug as well as shielding its effects on non-target cells. The accumulation of nanomedicines in tumor is a result of the enhanced permeability and retention (EPR) effect by means of improved circulation times. The leaky and permeable tumor vasculature in the absence of efficient lymphatic drainage system results in the superior accumulation of nanomedicines in tumor tissues (Rezvantlab et al., 2018). PLGA NPs targeting strategy involve either the active or passive targeting approach. In passive targeting, NPs enhance the drug accumulation in tumor by boosting the effect of the drug. This is done by increasing the stability and blood circulation time. For instance, the anticancer effect of curcumin is negated by the low aqueous solubility, instability, and reduced bioavailability. Curcumin-loaded PLGA NPs with additional surface coat of chitosan and PEG (CNPs) to achieve an optimum therapeutic effect was explored (Duruel et al., 2017). The study highlighted the superior characteristics of the CNPs with a better cytotoxicity effect, improved anti-migratory and anti-invasive properties, and ability to induce apoptosis in a metastatic pancreatic cancer model. Newer approaches involve combination therapies by combining chemotherapeutic drugs for enhanced effects. For example, curcumin and methotrexate were co-encapsulated in PLGA NPs, and their combined effects were investigated both *in vitro* and *in vivo* as a potential breast cancer therapeutic system (Vakilinezhad et al., 2019). The delivery system showed enhanced antitumor activities in combination when compared to single treatment of both drugs. Further, the formulation slowed down the growth of tumor and ultimately diminished the tumor altogether.

The absence of tumor-targeting moiety property of PLGA has resulted in various approaches being introduced to facilitate PLGA nanovehicles with tumor-targeting abilities which is also referred as active targeting (Bustos-Valenzuela et al., 2011). In this approach, PLGA NPs are attached to ligands (antibodies, aptamers, and peptides) which bind to receptors on tumor surface, promote cellular uptake, and enhance specificity. PLGA–star glucose NPs were synthesized for delivery of docetaxel (DCT-loaded PLGA–Glc). DCT-loaded PLGA–Glc NPs showed an improved anti-proliferation efficiency rather than control in HEp-2 model. These results suggest that NPs have the ability to actively target tumor established on interactions with glucose transporters and the hypoglycemic environments of the tumor. PLGA encapsulation of antitumor drug doxorubicin hydrochloride was reported by Zhao and coworkers (Ortega-Oller et al., 2015). Ahmed et al. (2019) performed the nanoencapsulation of the phenolics isolated from *Callistemon citrinus* plant into PLGA NPs and reported the enhanced bio-activity of the phenolics from *C. citrinus* against breast cancer cell lines, i.e., MDA-MB 231, MCF-10A, and MCF-7 by about two folds. Moreover, the addition

of berberine to phenolics-rich extracts of *C. citrinus* further increased their cytotoxic activities in free and encapsulated forms compared to phenolics-rich control extracts of *C. citrinus*. The results from their finding suggest that nanoencapsulation of anticancer compounds either alone or in combination with other bioactive compounds could be the important technique for cancer chemotherapy. The combination of anticancer drugs and polymer nanocarriers is highly regarded as an alternative approach for increased drug functionality with high stability and controlled release characteristics. Nonetheless, reproducibility in addition to complexity involved in cell signaling still poses a challenge to these promising drug carriers in clinical setting.

4.8 PLGA NPs FOR IMAGING

Molecular imaging is the detection method wherein functionalized probes encapsulated with contrast agents are administered to patients for disease diagnosis and detection. A study in 2012 demonstrated a method wherein high payloads of metallic gold nanocrystals were encapsulated into PLGA NPs. The encapsulation was carried out through a ligand–polymer and ligand–ligand esterification reaction that resulted in the production of lipid-PLGA NPs that were diagnostically active (Danhier et al., 2012). The result showed a system that had X-ray attenuation vastly greater to iodine-based contrast agents which were commercially available.

In addition, *in vitro* studies also established the usage of NPs as imaging probes in biological and medical applications. The superparamagnetic iron oxide (Fe_2O_3) NPs (SPIONs) have been used as extensively as highly sensitive imaging probes to improve the quality of magnetic resonance imaging (MRI) scan by providing contrast to it. PLGA NPs were conjugated with Herceptin for the targeting of human epidermal growth factor receptor 2 (Her2/neu) overexpressing in breast cancers (Dorati et al., 2017). This strategy was considered highly beneficial in improving the diagnostic and therapeutic efficacy for many cancer treatments. An amphiphilic Gd(III) complex loaded in PLGA NPs efficiently resulted in a highly sensitive MRI contrast and can be used as a novel tool in guided drug delivery applications (Duruel et al., 2017). The high stability and sensitivity of PLGA NPs allowed its *in vivo* accumulation in mice melanoma xenograft (Zhang et al., 2018a). Once loaded with drug and contrast agents, PLGA NPs and nanoemulsions can be proposed as efficient MRI and fluorescent imaging agents (Zhang et al., 2018a). These results show that the dyes would continue to remain encapsulated within the polymer for sufficient time so that the drug can reach any organ. In addition, the release is not affected even with a decline in pH produced by cell internalization. Therefore, the possibility of these NPs to be used as systemic imaging agents can be exploited in the future.

4.9 PLGA NPs IN BONE REGENERATION

The poor mechanic properties of PLGA have limited its application for fracture treatment especially in high load-bearing bones. Synchronizing the time taken for polymer degradation with that of tissue growth is an important feature in

designing scaffolds for bone regeneration. The hydrophilic nature of PLGA is due to the presence of glycolic acid have several advantages. The hydrophilic property of PLGA helps accelerate the polymer degradation as it speeds up polymer and scaffold wettability. Due to this, PLGA 75/25 is more compatible for tissue regeneration than PLGA 50/50 (Dorati et al., 2017). Bone morphogenetic growth factors (BMPs) are the most tested group of molecules. Several studies have reported the delivery of BMPs from PLGA NPs (Zhang et al., 2018a; Duruel et al., 2017). The size of NPs is generally larger in these cases in order to prevent the cellular uptake of PLGA NPs. This is due to factors like BMP2 initiates the signaling pathway from the cell surface by binding to two surface receptors, thereby requiring to be released into the extracellular medium (Bustos-Valenzuela et al., 2011). Bone regeneration by drug delivery can be improved by controlling the initial burst, a characteristic observed in most PLGA NPs used as drug carriers (Ortega-Oller et al., 2015). However, modifying the formulation to use more hydrophilic polymers has shown to reduce the initial burst and to deliver bioactive proteins over long time periods. Many data have now shown that combining PLGA scaffolds for tissue regeneration with drug delivery systems can be a suitable strategy to improve tissue regeneration. However, further work needs to be done to improve these systems to increase their efficiency. The recent applications of PLGA NPs for targeted drug delivery system are summarized in Table 4.1.

4.10 DEGRADATION OF PLGA

The degradation of poly(lactide-co-glycolide) occurs through the hydrolysis of its ester bonds which ultimately converts it into smaller subunits of glycolic acid and lactic acid. These subunits are endogenous to the body and therefore do not illicit any immune response or reaction (Singh et al., 2017). Poly(lactide-co-glycolide) degradation has been proposed to occur through a two-stage process which initially includes random hydrolytic cleavage of ester bonds which leads to the reduction of its molecular weight, and the second stage trails the initiation of loss in the mass of the polymer matrix which is further linked by an surge in the rate of chain scission. The studies have shown that relatively large-sized PLGAs degrade through a heterogeneous process, in which the core of the nanosphere degrades at a faster rate than the outer surface (Singh et al., 2017). The *in vivo* enzyme-catalyzed PLGA degradation has also been reported (Cai et al., 2003).

4.11 FUTURE PROSPECTS OF PLGA FORMULATIONS FOR CLINICAL APPLICATIONS

There are many recent developments in PLGA-based formulations in the areas of diagnosis and targeted therapies of human diseases (Martins et al., 2018; Danhier et al., 2012). A few PLGA-based pharmaceutical products have been approved for the human use such as Arestin®, Bydureon®, Eligard®, Lupron depot®, Ozurdex®, Signifor LAR®, Vivitrol®, and Zoladex® (Table 4.2).

TABLE 4.1
PLGA-Loaded Drug Used in Drug Delivery

Drug Loaded in PLGA NP	Main Targets	Methods	Application	References
Simvastatin (tetracyclin-PLGA)	Bone tissue	Solvent emulsification method	TC to PLGA copolymer conjugation can target bone and is used for the treatment of osteoporosis with SIM	Wang et al. (2015)
Paclitaxel	Microtubules	Solvent and extraction technique	Paclitaxel PLGA intermingled with vitamin E and tocopheryl polyethylene glycol succinate (TPGS) has neoplastic activity against primary ovarian carcinoma breast and colon tumors	Fateme et al. (2014)
Cisplatin	DNA adducts	Double-emulsion solvent evaporation method	Cisplatin in PLGA nanoparticles exhibited higher therapeutic efficacy on human prostate cancer LNCaP cells	Fateme et al. (2014)
Curcumin	Cytoplasmic proteins Vascular endothelial growth factor	Emulsion diffusion evaporation technique Double emulsion solvent evaporation technique	Nanoparticle encapsulation improves oral bioavailability of curcumin and was effective against metastatic ovarian, breast cancer, and prostate cancer cells PLGA-VEGF NP promotes fast healing due to the sustained and combined effects of VEGF and lactate, and it enhanced the proliferation migration of keratinocytes and upregulated the expression of VEGF R2 at mRNA level for active healing of non-diabetic and diabetic wounds	Anand et al. (2010) Cherreddy et al. (2015)
Dexamethasone (a glucocorticoid)	Cytoplasmic receptors	Solvent evaporation technique	Drug-loaded NPs have efficient suppression proliferation of vascular smooth muscle cells	Panyam et al. (2012)

(Continued)

TABLE 4.1 (Continued)
PLGA-Loaded Drug Used in Drug Delivery

Drug Loaded in PLGA NP	Main Targets	Methods	Application	References
Apigenin	Skin melanoma cell lines	Solvent displacement technique	Antiproliferative action against skin melanoma cell lines	Das et al. (2016)
Vancomycin, gentamicin, and lidocaine	Wound sites	PLGA-collagen nanofibrous membranes	Efficient delivery, prolonged local bioavailability, and enhanced pharmacological response	Chen et al. (2012)
FOL-PEG PLGA micelles loaded with DOX	Cancer cells	Nanoprecipitation and solvent evaporation technique	Higher cytotoxicity to the cancer cells	Zhang et al. (2014)
Berberine (PLGA-PEG NPS)	PCSK-9 for the treatment of LDL-cholesterol (HepG2)	Nanoprecipitation encapsulation	PCSK-9 for the treatment of LDL-cholesterol yielded significant decrease in the risk of mortality associated with hypercholesterolemia	Ochin and Garelnabi (2018)
Saponin β -aescin	J774 macrophages and non-phagocytic MRC-5 cells	Emulsification solvent evaporation technique	β -Aescin-loaded PLGA NPs (formulation VIII) exhibited reduced cytotoxicity of β -aescin while maintaining its antileishmanial efficacy	Van et al. (2011)
PLGA-PMA:PLA-PEG (PP) nanoparticles	Lymph nodes	Nanoprecipitation method	Lymphatic uptake and retention for prevention/treatment of oligometastases	Rao et al. (2010)
PLGA scaffold (fibroblast growth factor (bFGF))	Rotator cuff tear (RCT)	Emulsion electrospinning	bFGF-PLGA scaffold had better biocompatibility and biodegradability and a higher load-to-failure and stiffness leading to a more pronounced effect in the treatment of RCT	Zhao et al. (2014)
Mannosylated PLGA NP	Brain	Double-emulsion solvent evaporation technique	Efficient delivery of the drug into brain macrophages	Patel et al. (2018)

(Continued)

TABLE 4.1 (Continued)
PLGA-Loaded Drug Used in Drug Delivery

Drug Loaded in PLGA NP	Main Targets	Methods	Application	References
Co-delivery of docetaxel (DTX) with vitamin E TPGS	Multidrug resistance of the cancer cells	Nanoprecipitation method	The co-encapsulated TPGS (tocopheryl polyethylene glycol succinate) functions as a pore-forming agent for producing porous nanoparticles, higher drug, encapsulation efficiency and faster drug release, and also an active matrix component that could inhibit P-gp ATPase and drug efflux to overcome multidrug resistance of the cancer cells	Zhu et al. (2013)
PLGA-DTX (docetaxel) NP	Oral cancer	Nanoprecipitation method	Increased antiproliferative efficiency of PLGA DTX NPs compared with free DTX, in a dose-dependent manner against SCC-9 human tongue carcinoma cell line	Gupta et al. (2018)
Coumarin-6 PLGA NP	RWM into the inner ear	Emulsion solvent evaporation method	Showed enhanced therapeutic effects and understanding of RWM crossing processes of PLGA NPs	Zhang et al. (2018b)

(Continued)

TABLE 4.1 (Continued)
PLGA-Loaded Drug Used in Drug Delivery

Drug Loaded in PLGA NP	Main Targets	Methods	Application	References
Docetaxel-loaded M-PLGA-TPGS NPs	Breast cancer	Modified nanoprecipitation method	Docetaxel-loaded M-PLGA-TPGS NPs exhibited significantly superior anti-tumor effect compared with PLGA TPGS NPs and commercially available taxotere formulation	Tao et al. (2013)
PLGA-Hp55 NPs	Oral insulin	Emulsion solvent diffusion method in water	Drug release <i>in vitro</i> and hypoglycemic effects in diabetic rats were evaluated	Cui et al. (2007)
PLGA derivatized with the peptide g7 (g7-NPs) was loaded with loperamide and with a fluorescent dye, rhodamine-123108	Central nervous district	NA	The parent opioid peptides cross the blood-brain barrier by adsorption-mediated endocytosis by the brain capillary endothelial cells, due to their amphipathic character and helical conformation	Tosi et al. (2007)
(PLGA) NPs loaded with two potent ARVs, grifithsin (GRFT) and dapivirine (DPV)	Synergistic effect for HIV prophylaxis	Double emulsion solvent evaporation	Combination of GRFT and DPV in nanoparticles is highly potent and possesses properties critical to the design of a sustained release microbicide	Yang et al. (2019)

TABLE 4.2
Current Clinical Trials and Commercially Available PLGA-Based Therapeutics

Clinical ID/ Name	Condition/Diseases	Drug	Treatment	Status	References
NCT02487186	Chronic periodontitis	Doxycycline	Ultrasonic periodontal debridement treatment followed by local application of doxycycline-encapsulated PLGA microspheres	Clinical trial completed	https://www.clinicaltrials.gov/
NCT00836797	Preservation of alveolar bone height	–	Bone regeneration in alveolar sockets with PLGA scaffold	Clinical trial completed	https://www.clinicaltrials.gov/
NCT02568527	Limbal stem cell deficiency (LSCD)	–	PLGA scaffold as a substitute for using donor human amniotic membrane (hAM) for the treatment of LSCD	Clinical trial completed	https://www.clinicaltrials.gov/
NCT04094298	Rotator cuff disease	Triamcinolone acetonide	PLGA microspheres that slowly release triamcinolone acetoneide enabling their prolonged exposure to the point of injury	Clinical trial recruiting	https://www.clinicaltrials.gov/
NCT04619056	Soft tissue sarcoma	SN-38	PLGA drug loaded with SN-38	Clinical trial completed	https://www.clinicaltrials.gov/
Trelstar®	Prostate cancer	Triptorelin pamoate	Palliative treatment of advanced prostate cancer in which triptorelin pamoate microgranules are incorporated in a PLGA	recruiting/phase 1 2000	https://www.clinicaltrials.gov/ Zhong et al. (2018)
Risperdal Consta®	Schizophrenia and bipolar disorder	Risperidone	PLG microspheres encapsulated with risperidone for treatment by deep intramuscular (IM) deltoid or gluteal injection	2003	Park et al. (2019)
Signifor LAR	Cushing's disease and acromegaly	Pasireotidepamoate	Pasireotide pamoatedrug uniformly distributed within microspheres of PLGA	Approved 2014	Park et al. (2019)
Lupaneta Pack™	Endometriosis	Leuprolide acetate	Microparticles can last up to three months	Approved 2012	Park et al. (2019)

(Continued)

TABLE 4.2 (Continued)
Current Clinical Trials and Commercially Available PLGA-Based Therapeutics

Clinical ID/Name	Condition/Diseases	Drug	Treatment	Status	References
Triptodur Kit®	Central precocious puberty	Triptorelin pamoate	Extended release formulation that consists of a biodegradable polymer with segments of PLG and PLGA	Approved 2017	Zhong et al. (2018)
Arestin™	As adjunct in adult periodontitis	Minocycline	PLGA microspheres, embedded with Arestin and administered through periodontal route	Approved 2001	Kim et al. (2019)
Bydurcon™	Type 2 diabetes mellitus	Exenatide	Only long-acting exenatide product in the market which is in microsphere	Approved 2012	Cai et al. (2013)
Zilretta™	Osteoarthritis	Triamcinolone acetonide (TA)	A once-weekly PLGA microsphere injection	Approved 2017	Park et al. (2019)
Sublocade™	Moderate to severe opioid use disorder	Buprenorphine	TAs are embedded in a PLGA matrix to provide extended pain relief	Approved 2002	Ghitmanet al. (2020)
Perseris™	Schizophrenia	Risperidone	buprenorphine encapsulated in PLGA NPs	Approved 2018	Blasi et al. (2019)
			PLGA and risperidone are mixed just before injection to avoid the degradation of polymer matrix by risperidone		
			Release drug for up to one month		

Despite the large volume of data available for PLGA NPs as drug delivery vehicles, its transition from research labs to an efficient therapeutic option is faced with many hurdles. Though most of the pre-clinical data are promising, most of the vehicles become unresponsive when examined in clinical studies. Size optimization and drug loading and release kinetics are factors that need to be constantly evaluated as batch-to-batch variation and reproducibility are major concerns that need to be addressed. Other major drawbacks for the clinical translation of PLGA-based NPs are the initial uncontrolled release of loaded drug (high initial burst), production of acidic products in the system after its complete bio-degradation, difficulty in scaling up process to industrial levels, and most importantly their nanotoxicology (Sharma et al., 2016). The dedicated studies are required to understand the toxicology of the degraded products of PLGA which are lactic acid and glycolic acid to the major organs and tissues of human beings (Navarro et al., 2016). Another factor that could influence the clinical transition of PLGA NPs is the high inter- and intra-individual heterogeneity of the EPR effect (Rezvantab et al., 2018). Researchers need to focus on a rational design wherein all these factors are addressed from the initial synthesis process itself while focusing on reproducibility and how the EPR effect could impact accumulation, penetration, distribution, retention, and efficacy of NP formulations.

4.12 CONCLUSION

The preparation of PLGA NPs has advanced from traditional methods to newer more advanced processes where drug loading is higher with higher specificity. PLGA NPs have been widely explored for carriers and localized delivery of therapeutic agents. PLGA NPs have shown to be the most effective carriers in drug delivery systems, through repeated success in multiple reports. A quantum property such as low critical aggregation concentration (CAC) and large surface area of the NP's not only increase the stability but also prolongs its circulation time within the body. The protocol for the preparation of PLGA NPs can be modified by optimizing the homogenization process, type of surfactant used, and surface modification to alter its targeting behavior with optimum property enhancement. By modifying its synthesis, its properties such as hydrophobicity, release profile, and target moiety can be tailored to specific properties which can cater to a wide range of diseases. The endless possibilities of PLGA as drug delivery agents are expanding with new drugs and add-ons which further advances its applicability, although a long road lies ahead to convert it into a faithful practical addition for the next generation of drug-delivery systems for clinical use.

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5 Employing New Targeted Nanoencapsulation for Alzheimer's Disease Treatment *A Change for the Better?*

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5.1 INTRODUCTION

5.1.1 HISTORICAL VIEWPOINT AND PRESENT PICTURE OF ALZHEIMER'S DISEASE (AD)

In the 19th century, a woman died of a disease that affected her cognitive functions. In 1906, Alois Alzheimer, a German physician, first reported that this disease was due to abnormalities in the brain cells, and hence this disorder was named after his name. The reason behind this abnormality is dense deposition on the outer surface and curled fibers on the inner side of nerve cells. Now, this disease affects several people worldwide, e.g., more than 3.2 million persons in the Europe and more than 5 million persons in the United States. The major trademarks of Alzheimer's disorder are plaques that are rich in amyloid curled nerve fibers and degeneration of the nerve cells, and these symptoms slowly and steadily lead to death. One of the significant factors for the prominence of this disorder is age, because over 65 or 85 years, the prevalence of this disorder increases by approximately 35%. Some of the other probable dangers include hypertension, high sugar level, brain injury, cardiovascular diseases, cancer, hypercholesterolemia, obesity, ApoE (apo-lipoprotein E), poor education, and exposure to toxic materials that are harmful to the brain such as heavy metals, copper, and zinc. A number of improvements have been made for the effective management of this disorder, but still this disorder is devastating for patients. However, these advancements have increased the lifespan of these patients.

Consequently, the improvement in the existing techniques is urgently required to do early diagnosis, and there is the need for reliable systems for delivering the drug, so that it can act on specific target sites.

5.2 DRUGS BASED ON NANOPARTICLES FOR ALZHEIMER'S DISEASE

The use of nanoparticles has been dramatically increased in the field of nanotechnology. Nanoparticles were developed and used as targeted drugs; this hypothesis

was given by the magic bullet concept of Paul Ehrlich. There are some applications based on nanoparticles in the medical field, and these came to the world in the time between 1960 and 1970 and are used for the effective delivery of vaccines and drugs. Nowadays, nanoparticles are used as diagnostic as well as therapeutic agents (Kreuter, 2007). In preclinical studies, the use of nanoparticles has been analyzed as therapeutic agents. But due to toxicity issues, their use has been restricted on humans (Dai et al., 2020). Acrylamide nanoparticles were used very first time, but now a number of compounds that are biologically reliable and can be degraded biologically are used in the medical field intensively. Incorporation of drugs into nanoparticles can be done at the time of processing and can be adsorbed on the surface of already-formed nanoparticles. Nanoparticles are very beneficial in terms of their way of delivering the drugs as they can be delivered intravenously, they can be surface engineered, a broad range of drugs can be delivered using them, and they are very efficient in crossing the barriers, e.g., blood–brain barrier (Gao, 2006).

5.3 TYPES OF NANOPARTICLE-BASED DRUGS

5.3.1 TACRINE

Tetrahydroaminoacridine is the chemical name of tacrine (Figure 5.1). The function of this drug is based on inhibiting acetylcholinesterase non-selectively and reversibly (Adem, 1990). Tacrine was the first drug for the symptomatic management of Alzheimer's disorder permitted by Food and Drug Administration in 1993. This drug has been reported as potent inhibitor of enzyme acetylcholinesterase (Hakansson, 1993). But this drug has been stopped as it is very toxic for the liver and is less available biologically (Stachlewitz et al., 1997). Tacrine is less available biologically because the drug is significantly reduced in the intestine and liver and its half-life is short. But by administering the drug intravenously or by any other way can decrease its toxic effect on the liver as well as issues related to its availability in biological tissues. Large surface area and maximum absorption of the drug can be provided by delivering the drug by nostrils, and this also reduces the reduction of drug in the intestine and liver (Luppi, 2011).

5.3.1.1 Mode of Action of Tacrine

It was found to obstruct the enzyme acetylcholinesterase (Figure 5.2). In short, this drug is a reversible cholinesterase inhibitor, and it also interacts with cholinergic

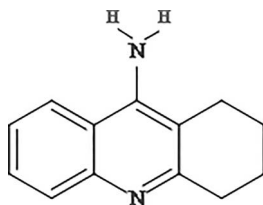


FIGURE 5.1 Structure of (1,2,3,4-tetrahydro-9-acridinamine) tacrine compound CID: 1935.

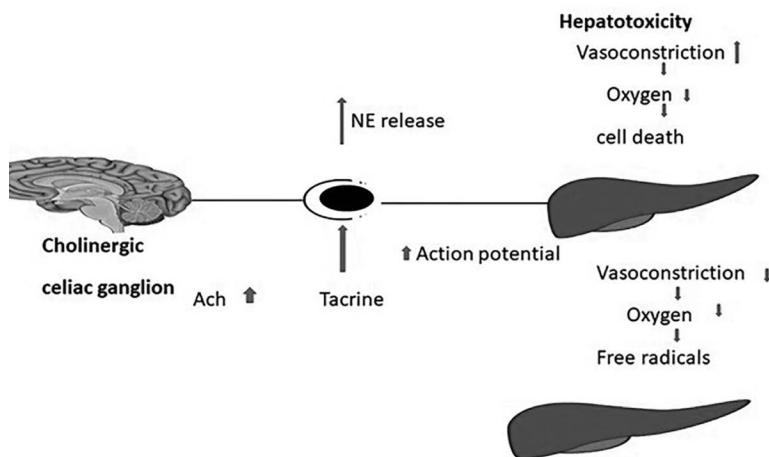


FIGURE 5.2 Mechanism of action of tacrine in the brain and hepatotoxicity in the liver. Tacrine interacts with cholinergic receptor and helps in the release of neurotransmitter which in turn enhances brain function. Tacrine shows severe hepatotoxicity in the liver.

receptors, interacts with sodium and potassium channels, and helps in the release and synthesis of neurotransmitters which helps in the treatment of Alzheimer's disease (Luppi, 2011).

Unluckily, the main side-effect of the drug is on the liver, which ultimately leads to cell death which in turn limits its use in the treatment of the disease, and hence, this drug is withdrawn (Table 5.1). Tacrine inhibits the breakdown of acetylcholine in the nerve tissues present in the abdomen which enhances the involuntary movement in the liver which leads to tightening of the blood vessels and ultimately leads to hypoxia and liver cell death (Emerson, 1993).

5.3.1.2 Efficacy of the Drug

It was reported that at a dose of 80–160 mg/day, there was improvement in intellectual and behavioral insufficiency in a number of patients suffering from neurodegeneration.

5.3.1.3 Adverse Effects

Limitations associated with the therapy are the smaller number of recoveries and high vulnerability to liver side-effects (Wagstaff, 1994).

5.3.2 RIVASTIGMINE

Both the enzymes acetylcholinesterase and butyrylcholinesterase were noncompetitively and reversibly inhibited by the drug rivastigmine (Figure 5.3a). This was approved by the Food and Drug Administration in 2000 as a means of effective treatment of Alzheimer's disease. It was studied that the use of specific medicine in the form of nanoparticles leads to an increase in absorption by a number of

TABLE 5.1
Effect of Nanoparticle-Based Drug on Alzheimer’s Disorder

Name of Drug	Nanoparticles	Category of Drug	Method of Preparation	Animals Used	Study Outcome	Clinical Phase (https://clinicaltrials.gov/ct2/)
Tacrine	Albumin nanoparticles	ChEI	Coacervation		Tacrine nanoparticles proved good access across sheep nasal mucosa (Luppi, 2011).	Withdrawn (Grasing, 2009)
Rivastigmine	Chitosan nanoparticles	ChEI	Ionic gelation	Rats	Rivastigmine is increased significantly in the brain by intranasal administration of nanoparticles (Fazil, 2012).	Completed (Aguilar, 2014)
D-Penicillamine		Metal ion chelator			Amyloid- β protein is solubilized by D-penicillamine released from the nanoparticles (Cui, 2005).	
Clioquinol	PBCA nanoparticles	Metal ion chelator		Transgenic mice (APP/PS1)	Retention of radiolabeled 125I clioquinol nanoparticles in the brain was significantly more when compared with controls (Kulkarni, 2010).	Completed (Grolez, 2015)
VEGF	PLGA nanoparticles		Double emulsion solvent evaporation	Transgenic mice (APP/PS1)	VEGF-loaded PLGA nanoparticles prevented loss of neurons, and helps in reducing abnormalities related to cerebrovascular and beta-amyloid clearance (Grasing et al. 2009; Herrán, 2013).	
EGCG	Phenol derivative nanolipidic particles		Co-solubilization	Rats	Nanolipidic particles help in increasing the alpha-secretase action of EGCG (Religa, 2013).	Phase 3 (D Skaper, 2017)
Estradiol	Estrogen PLGA nanoparticles		Emulsion–diffusion–evaporation	Rats	Orally administered drug nanoparticles with TweenR 80 were effective (Ohkura, 1995).	Completed (Schmidt, 2015)
Lactoferrin	PEG–PCL nanoparticles	Chelate metal ions	Emulsion/solvent evaporation	Rats/mice	Brain function of rat was considerably enhanced by delivering the nanoparticles conjugated with lactoferrin in the Morris water maze task (Epperson, 2012).	Phase 2 (Jererny et al.)
Quercetin	PBCA	Free radical scavenger	Anionic emulsion polymerization	Wistar rats	PBCA-coated quercetin shows increased bioavailability (Bagad, 2015).	Early phase 1 (Yang, 2005)

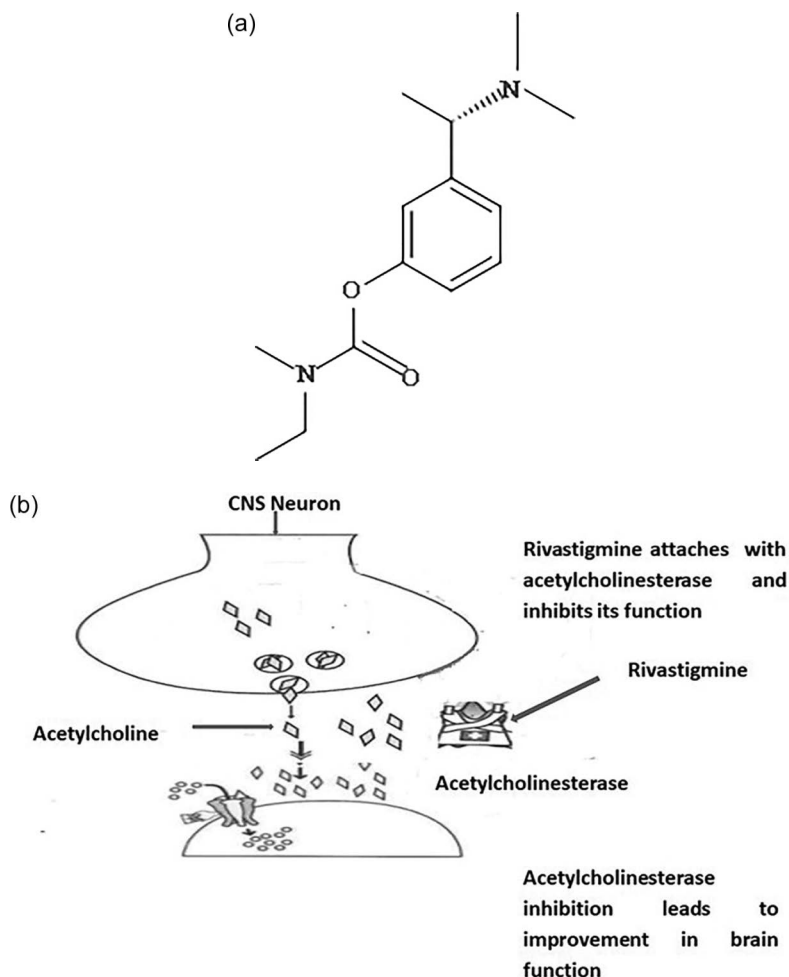


FIGURE 5.3 (a) Chemical structure of rivastigmine compound CID 77991. (b) Mechanism of action of rivastigmine in the brain. Rivastigmine attaches with acetylcholinesterase and inhibits its function which ultimately leads to the release of neurotransmitter and improves brain function.

factors as compared with free drugs. Administration of rivastigmine nanoparticles through the route of nasal cavities leads to increased concentration of the medicine in the brain (Fazil, 2012). The nanoparticles used for the coating of rivastigmine are poly(butylcyanoacrylate) nanoparticles (Joshi, 2010). Overexpression of MARK4 (microtubule affinity-regulating kinase 4) is also responsible for Alzheimer's disease, and it can be used as a potential target for the treatment of this disease. In a recent study, rivastigmine tartrate has been reported to inhibit the expression of MARK4 (Shamsi et al., 2020a). In another study, it has been shown that

MARK4 can also be regulated with the help of cholic acid (Anwar et al., 2020). In a recent study, rivastigmine tartrate has also been shown to form a complex with human transferrin which is very significant for the therapy of Alzheimer's disease (Shamsi et al., 2019). In another study, rivastigmine has been reported to form a good complex with human serum albumen (HSA) which can also prove as a good therapeutic agent as HSA can be used as a nanocarrier for the sustained release of rivastigmine (Shamsi et al., 2020b).

5.3.2.1 Mechanism of Action

Rivastigmine also acts as an inhibitor of acetylcholinesterase and specifically inhibits the enzyme present in the brain, but it least inhibits the enzyme present in the peripheral nervous system (Figure 5.3b). G1 acetylcholinesterase is the predominant form of the enzyme present in the brain, which is mainly inhibited by rivastigmine. In contrast to other acetylcholinesterase inhibitors, it does not affect liver cytochrome-450 as it is not metabolized by the liver, and hence, it is not hepatotoxic (Polinsky, 1998).

5.3.2.2 Efficacy of the Drug

This drug is less interactive with other drugs, which is a great benefit of the drug as patients in old age have to undergo a number of medications for managing other diseases (Table 5.1).

The hepatic system is not involved in the inactivation and elimination of the drug, and so, it will cause no ill effects on the liver.

Behavioral insufficiency is retained.

5.3.2.3 Adverse Effects of the Drug

Minimum side effects are encountered as there is no adverse effect on the liver.

Headache and dizziness are the main side effects (Darvesh, 1998).

5.3.3 D-PENICILLAMINE

The drug D-penicillamine (Figure 5.4a) can be easily released from the nanoparticles as it is conjugated via thioether bond with dithiothreitol which in turn is a reducing agent. After the release, D-penicillamine is capable to redissolve the aggregate of copper amyloid protein consequently with the help of nanoparticles. D-penicillamine can be easily administered into the brain so as to prevent the formation of amyloid protein (Cui, 2005).

5.3.3.1 Mode of Action

Studies found that with ageing and mainly in neurodegeneration, there is a deposition of metals, e.g., copper, zinc, and iron. Interaction of copper, zinc, and iron with amyloid β favors the development of neurodegenerative diseases. D-penicillamine is a metal chelator, and it chelates with copper to reduce the free radical stress in the nervous system and reduces the generation of reactive oxygen species (Figure 5.4b) (Cui, 2005).

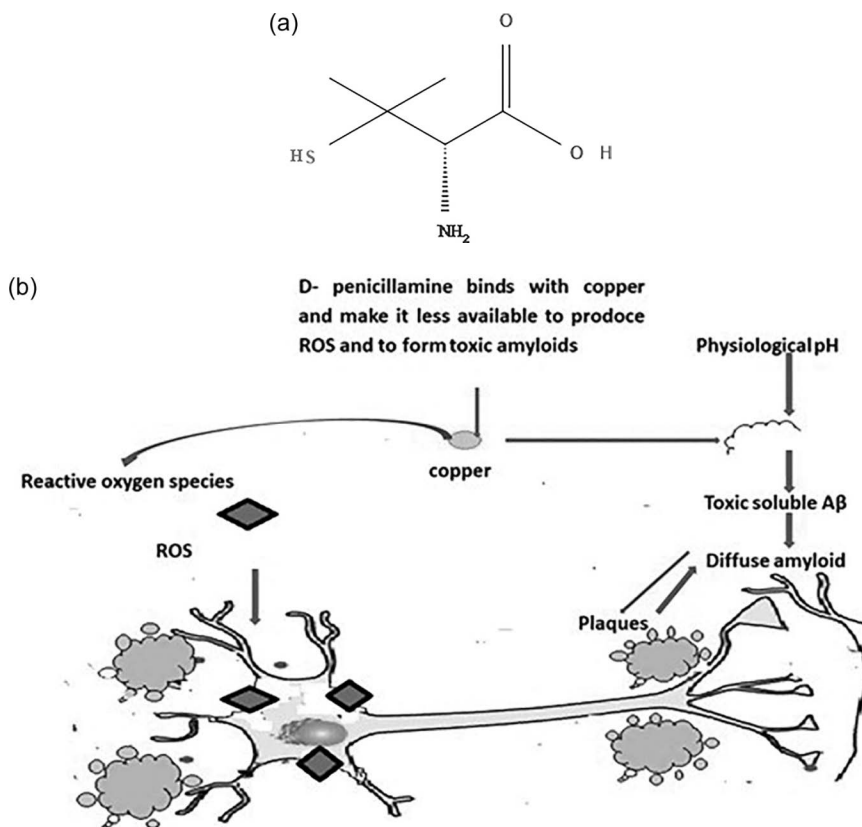


FIGURE 5.4 (a) Chemical structure of D-penicillamine, 2-amino-3-mercaptopropanoic acid, and 3,3-dimethyl-DL-cysteine compound CID 5852. (b) Mechanism of action of D-penicillamine. D-penicillamine helps in chelating inorganic ions, attaches with copper, makes it less available to produce ROS, and ultimately lowers the production of β -amyloid (Table 5.1).

5.3.3.2 Efficacy of the Drug

It was reported that treatment with D-penicillamine helps in reducing the extent of oxidative stress, but there is no improvement in intellectual function (Squitti, 2002).

5.3.3.3 Adverse Effects of the Drug

Severe allergies have been reported of this drug. Severe allergic thrombocytopenia has been reported in a 53-year-old lady.

Side effects on the kidneys were also reported.

Long-term usage of this drug leads to allergic rashes and depletion of platelets (Hennemann, 1975).

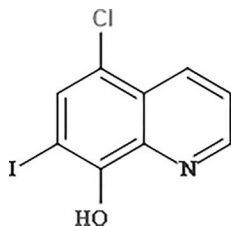


FIGURE 5.5 Structure of (5-chloro-7-iodoquinolin-8-yl) 2-iodochlorohydroxyquin chineform) clioquinol compound CID 2788.

5.3.4 CLIOQUINOL

Clioquinol (Figure 5.5) is the drug used as a tool for the identification of Alzheimer's disease. The nanoparticle used for loading this drug is poly(*n*-butyl-2-cyanoacrylate), and it is radiolabeled with ^{125}I . It was reported that administering the drug with the help of nanoparticles leads to effective transport into the brain and also increases the retention time in amyloid aggregates. When compared with free drug, radiolabeled nanoparticles of ^{125}I -clioquinol are more effective and their uptake was more (Kulkarni, 2010).

5.3.4.1 Mechanism of Action

Clioquinol is a metal-chelating agent, and it can chelate zinc and copper. Alzheimer's disease has the characteristic feature of the presence of toxic beta-amyloid and abnormal tau proteins which are stabilized by copper and zinc. Hence, if there is a method to bind these metals, then it becomes easy to dissolve the toxic amyloid proteins. It was reported that clioquinol binds with zinc and copper and destabilizes the toxic amyloid proteins (Bareggi, 2012).

5.3.4.2 Efficacy of the Drug

Noteworthy decrease in the deposits of beta-amyloid in transgenic mice was observed.

5.3.4.3 Adverse Effects

When clioquinol was administrated, systemically severe inflammation in the brain was reported (Zhang, 2013).

5.3.5 VEGF (VASCULAR EPIDERMAL GROWTH FACTOR)

Alzheimer's disease is caused by changes in blood circulation in the brain, and this is supported by evidences. Decrease of cerebral perfusion has been monitored in early stages of diagnosis, which continues up to ending phases of Alzheimer's disease. VEGF (vascular epidermal growth factor) (Figure 5.6a) is mainly concerned with protection of the nervous system, development of new blood vessels, and growth of axons. On the other hand, VEGF has very short life span and gets

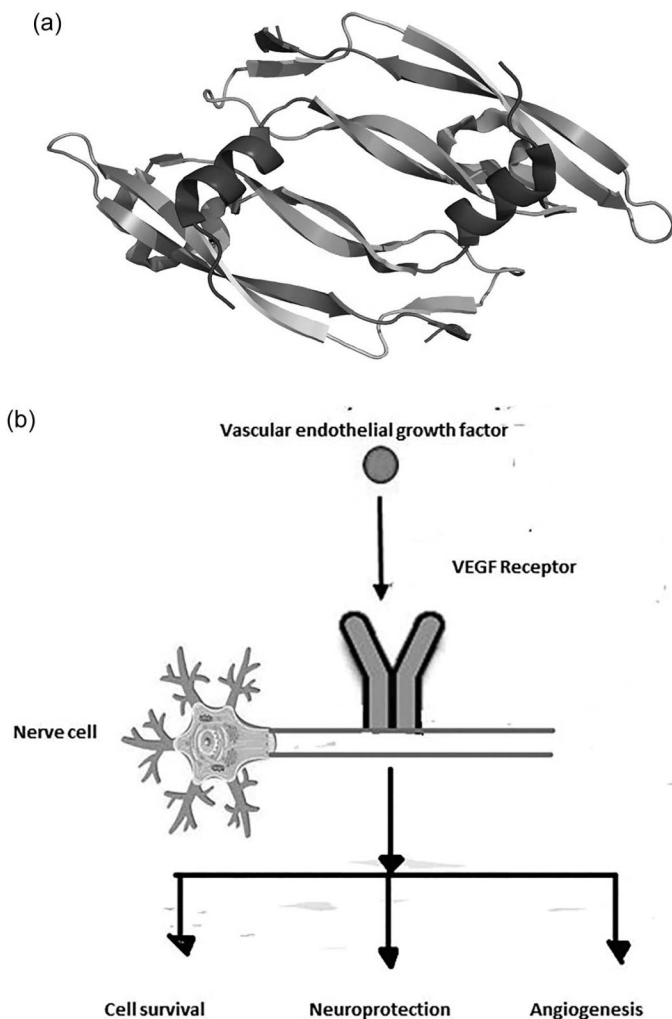


FIGURE 5.6 (a) VEGF (vascular endothelial growth factor) crystal structure (PMID: 16616187) (Shibuya, 2013). (b) Mechanism of action of VEGF. It is a growth factor; therefore, when it binds on nerve cells, it leads to cell survival, neuroprotection, and formation of new blood vessels.

degraded very easily, so its use is limited. But by using nanoparticles, its efficacy can be increased manyfold. It was reported that when VEGF is introduced in PLGA nanospheres, the efficacy of the drug in terms of neuroprotection increases in the nerve cells, and there is decrease in toxicity caused by amyloid aggregates. Additionally, it was reported that nanoparticles containing VEGF help to degrade aggregates of amyloid, defects in blood circulation are minimized, and nanoparticles halt the loss of nerve cells (Herrán, 2013).

5.3.5.1 Mechanism of Action

VEGFR-1 and VEGFR-2 tyrosine kinase receptors are activated when VEGF transmits a signal that leads to cell survival, neuroprotection, and angiogenesis (Figure 5.6b) (Rosenstein, 2010).

5.3.5.2 Efficacy of the Drug

Cognitive functions are restored in patients with Alzheimer's disease. It was also reported to improve circulatory functions (Religa, 2013).

5.3.6 EPIGALLOCATECHIN-3-GALLATE (EGCG)

In prediagnostic stages, epigallocatechin-3-gallate (Figure 5.7a) helps in preventing the formation of amyloid aggregates.

On the other hand, the major issues are biological availability of the drug and implication in crossing the barrier between the brain and the blood. Again, the problem can be resolved by incorporating the drug into nanoparticles, i.e., nano-lipidic (–)-epigallocatechin-3-gallate. It was reported that after incorporating into nanoparticles, biological availability of the drug increased manifold (Smith, 2010). Coating drugs with PLA–PEG nanoparticles helps in controlled release of the drugs (Singh, 2018). It was earlier reported that neurobehavioral changes induced by aluminum chloride in rats can be recovered by epigallocatechin-gallate (EGCG)-loaded nanoparticles (nanoEGCG) (Chang, 2015).

5.3.6.1 Mode of Action

EGCG is responsible for the stimulation of the PKC pathway (Figure 5.7b) (Levites, 2003). PKC (protein kinase C) is considered in the well-being of cells (Berra, 1997). EGCG activates this signaling pathway by excessive phosphorylation of PKC (Levites, 2003). Amyloid precursor protein can be processed via two pathways. In the first pathway, APP (amyloid precursor protein) processing is dependent upon beta- and gamma-secretase and is responsible for A β formation which ultimately leads to pathogenesis (Alzheimer's disease). In the other pathway, α -secretase process APP forms non-toxic sAPP. This α -secretase can be activated by EGCG via the PKC pathway (Levites, 2003). The PKC activation pathway is also responsible for the degradation of proapoptotic Bad gene (Berra et al., 1997).

5.3.6.2 Efficacy of the Drug

Buildup of β -amyloid in neurons can be decreased by EGCG (Kalfon, 2007).

Apoptosis of the cell, free radical-induced stress, beta-amyloid buildup, and redness can be suppressed with the help of EGCG (Zhang, 2017).

5.3.7 ESTRADIOL (FIGURE 5.8A)

Deficiency of intracellular estrogen is the leading cause of Alzheimer's disorder in females after post-menopause. So, it was reported that if estrogen is replaced by alternate synthetic hormonal therapy, the risk of the disease can be minimized

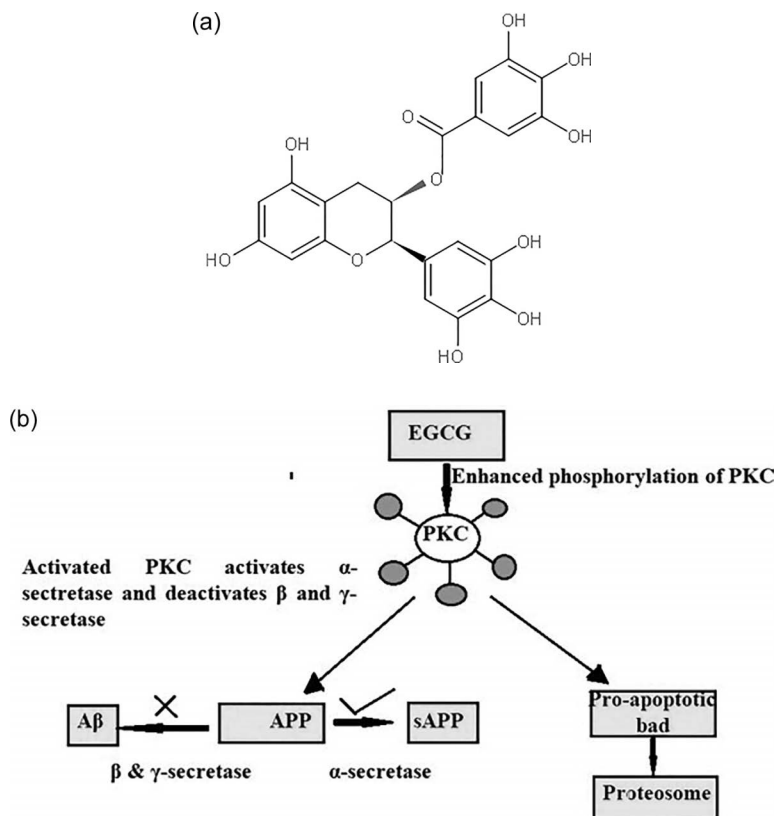


FIGURE 5.7 (a) Chemical structure of EGCG (epigallocatechin 3-gallate) compound CID: 65064. (b) Mechanism of action of EGCG. It leads to enhanced phosphorylation of PKC (protein kinase C) ultimately responsible for the stimulation of α -secretase pathway and inhibits β - and γ -secretase pathway.

(Smith, 2010). Nanoparticles that are loaded with PLGA and also coated with Tween R 80 show enhanced medicine accumulation in neurons. Biological availability of the coated medicine increases many times in the brain, so it was concluded that the disease can be prevented effectively by nanoparticle drugs (Ohkura, 1995).

5.3.7.1 Mechanism of Action

Females are mostly affected by Alzheimer's disease as compared to males. At menopause, the level of estrogen goes down, which can be a cause of the disease in females (Mittal, 2011). Estrogen therapy can improve symptoms of Alzheimer's disease via three pathways. First, estrogen interacts with ApoE and decreases its secretion, which leads to decreased deposition of β -amyloid as ApoE is responsible for the reduced clearance of β -amyloid (Figure 5.8b) (Paganini-Hill, 1996). Another pathway is the increased expression of acetylcholine transferase that

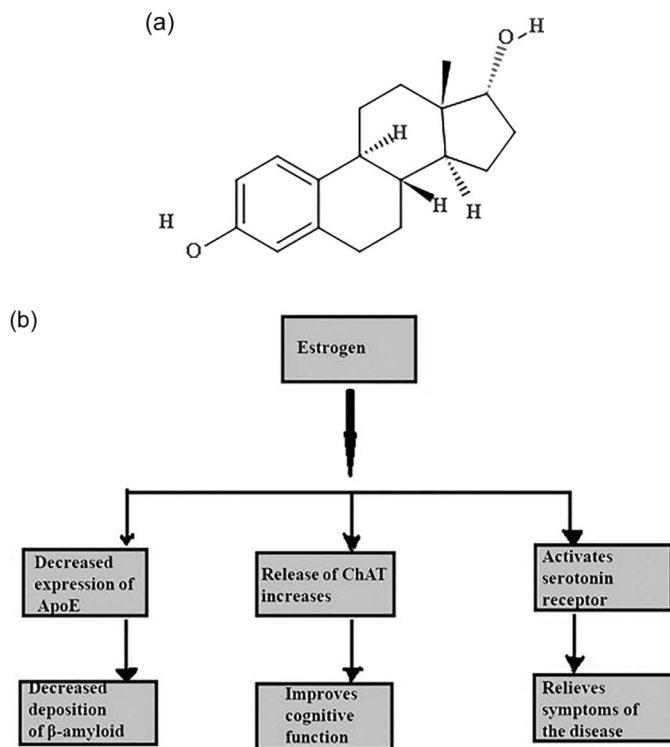


FIGURE 5.8 (a) Chemical structure of estradiol (17 alpha-estradiol) compound CID: 5757. (b) Mechanism of action of estrogen. Estrogen decreases the expression of ApoE, the release of enzyme acetylcholinesterase increases, and activated serotonin receptor ultimately leads to improved brain function.

helps in the synthesis of neurotransmitter and ultimately improves cognitive function (Bales, 1999). Third mechanism is the activation of serotonin receptor signaling which ultimately relieves stress level and decreases affective disorder (Emerson, 1993).

5.3.7.2 Efficacy of the Drug

Estrogen therapy is highly efficient to restore the intellectual functions in females suffering from Alzheimer's disorder. It also helps reduce the risk of disease development in healthy women. Additionally, it was shown in clinical trials that other cognitive functions such as memory and learning can also be improved by this therapy (Epperson et al., 2012).

5.3.7.3 Lactoferrin

Lactoferrin is a protein that can bind with iron with a molecular weight of 80 KD (Figure 5.9a). Lactoferrin was first purified by Sorenson and Sorenson in 1940 (Sorensen and Sorensen, 1940). Endothelial cells of the brain contain transferrin

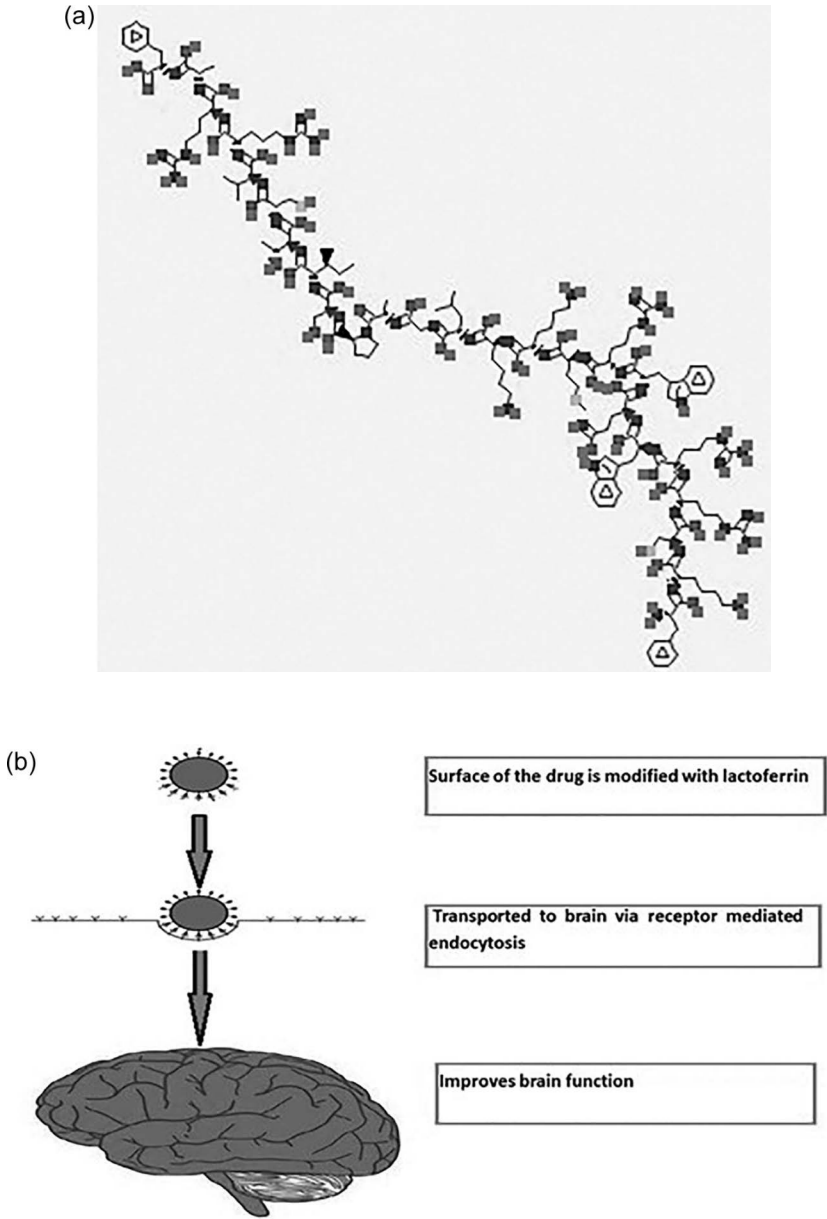


FIGURE 5.9 (a) Chemical structure of lactoferrin compound CID: 126456119. (b) Mechanism of action of lactoferrin-modified drug. Lactoferrin is a carrier molecule that can pass easily through the barrier between the brain and the blood with the help of receptor-mediated endocytosis. The drug for the management of the neurodegenerative ailment modified with lactoferrin and effectively delivered in the nervous system ultimately improves brain function.

receptors and are highly expressed here. Lactoferrin is an iron-binding glycoprotein and also belongs to the transferrin family. Patients with Alzheimer's disorder overexpress this protein, so it can be used as an appropriate carrier for the delivery of nanoparticles-loaded drug in the brain. When nanoparticles containing lactoferrin were prepared and administered by the nasal route, they improved the behavior of mice (Liu, 2013).

5.3.7.4 Mechanism of Action

It was reported that lactoferrin remained in the cerebrospinal fluid of the brain when administered intravenously. Lactoferrin is transported to the brain via choroid plexus, which is the main route for transportation (Meng, 2018). The barrier between the brain and the blood shields the nervous system from unwanted molecules, but lactoferrin is transported to the brain with the help of receptor-mediated endocytosis (Kamemori, 2008). It was reported that the expression of lactoferrin increases in response to inflammatory pathways in Alzheimer's pathology (Fillebeen, 1999). So, it is practiced as a transporter to carry the nanoparticle-loaded drug to manage the ailment as shown in Figure 5.9b (Kawamata, 1993).

5.3.7.5 Efficacy of the Drug

Lactoferrin is used as an active transporter for the delivery of drugs to the nerves. It was reported that Huperzine A (HupA) coated with PLGA nanoparticles and surface-modified by lactoferrin was found to be very efficient for delivering the drug to the brain through the intranasal route (Liu, 2013).

5.4 CONCLUSION

Alzheimer's-induced animal models can be successfully treated with targeted nanoparticles-based therapies. Rivastigmine–chitosan nanoparticles seem to be the best candidate for the treatment of Alzheimer's disease. Efficiency of targeted nanoparticles can be enhanced by understanding the mechanism of Alzheimer's disease.

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6 Nanomaterials-Assisted Elicitation of Pharmaceutically Important Secondary Metabolites from *In Vitro* Plant Cell Cultures

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6.1 INTRODUCTION

Materials or particles having a size ranging from 1 to 100 nm are categorized as nanomaterials (Chung et al. 2018a; Jeevanandam et al. 2018). Nanomaterials have gained importance in recent years owing to their potential applications in varied areas of domestic, industrial, and agricultural sectors (Marslin et al. 2017). In fact, nanomaterials have permeated in almost every discipline of science and technology (Anjum et al. 2019). Broadly, nanomaterials are categorized into the following types: (i) organic (e.g., dendrimers, micelles, liposomes, and ferritin); (ii) inorganic—(a) metal-based (e.g., Au, Ag, and Fe) and (b) metal oxide-based (e.g., aluminum oxide (Al_2O_3), cerium oxide (CeO_2), iron oxide (Fe_2O_3), magnetite (Fe_3O_4), silicon dioxide (SiO_2), titanium dioxide (TiO_2), zinc oxide (ZnO)); and (iii) carbon-based (e.g., fullerenes, graphene, carbon nanotubes (CNTs), carbon nanofibers, carbon black, and activated carbon) (Ealias and Saravanakumar 2017). Owing to their growing use in nanobiotechnology, they have emerged as potent agents to elicit secondary metabolites from *in vitro* plant cell cultures (Sharafi et al. 2013; Ghorbanpour and Hadian 2015; Hatami et al. 2016). Secondary metabolites are chemical compounds that are not vital for survival but help interact organism with its environment (Pagare et al. 2015). These are synthesized during plant adaptation to unfavorable environmental states (biotic and abiotic stresses). Besides this, secondary metabolites are also synthesized by microorganisms (bacteria and fungi) living inside plants' body as endophytes. Endophytes are a group of bacteria or fungi that colonize the inter- and/or intracellular spaces of plants (Gouda et al. 2016). They serve as rich repository of low-molecular-weight organic compounds which are of great economic value. Among the various approaches to produce secondary metabolites, elicitors have been employed using *in vitro* plant cell cultures (Anjum et al. 2019). Nanomaterial-assisted elicitation has provided a novel approach to harness secondary metabolites (Baiazidi-Aghdam et al. 2016). Presently, varied types of nanomaterials have been used as elicitors to derive secondary metabolites from *in vitro* plant cell cultures (Bhat and Bhat 2016; Sanchez-Munoz et al. 2019). However, among the nanomaterials used, silver, gold, copper, zinc, zinc oxide, copper oxide, cadmium oxide, aluminum oxide, silica oxide, cesium oxide, nickel oxide, and magnesium oxide have been used widely (Anjum et al. 2019). *In vitro* plant cell cultures provide a feasible approach to extract phytochemicals, which is not possible by chemical synthesis (Nandagopal et al. 2018). Although there are reports which have mentioned the use of nanomaterials as elicitors for enhanced production of secondary metabolites by increasing the expression of concerned genes (Ghasemi et al. 2015; Yarizade and Hosseini 2015), a comprehensive effort is required to figure out the various factors associated with biosynthesis of secondary metabolites mediated by nanomaterials (Hatami et al. 2018).

6.2 NANOMATERIAL-MEDIATED ELICITATION OF PLANT SECONDARY METABOLITES MECHANISM

Precise molecular details of the interplay of nanomaterials with biological surfaces are not clear widely. Nel et al. (2009) opined that carbonaceous nanomaterials

adsorb on cell surfaces through biophysical interactions. A range of biophysical interactions (hydrogen bond, electrostatic, hydrophobic, molecular recognition, metal-coordinate, and stereoselective) take place upon the contact of nanomaterials in biological environment (Wang et al. 2019). They enter plant cells by forming an envelope which is formed at the surface of the cells followed by formation of clusters which leads to changes in metabolic processes. Elicitors (nanomaterials) lead to activation of transduction cascades in plant cell cultures which in turn activate concerned enzymes responsible for biosynthesis of secondary metabolites (Ponti et al. 2010). Ghorbanpour and Hadian (2015) observed that enzymes like phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase are produced when exposed to nanomaterials during biosynthesis of secondary metabolites. They found that exposure of multi-walled carbon nanotubes (MWCNTs) at 100 and 250 $\mu\text{g/ml}$ positively correlates with the production of phenylalanine ammonia lyase. Similarly, Jalalpour et al. (2014) reported that production of total phenolics in *in vitro* culture is correlated with increase in phenylalanine ammonia lyase activity. Khodakovskaya et al. (2011) opined that multiple different genes are activated owing to exposure by nanomaterials (carbon based), which leads to changes similar to responses generated by biotic factors (insects, herbivores, or pathogens attack). It is noted that owing to elicitation by nanomaterials, reactive oxygen species like superoxide hydroxyl radical and hydrogen peroxide are produced in plant cells, which may be playing a role in production of secondary metabolites. Low and Merida (1996) observed that enzymatic and non-enzymatic antioxidant defense systems are regulated by oxidative bursts. Furthermore, according to Jabs et al. (1997), the redox status of plants might be altered due to generation of hydrogen peroxide which may be playing a role in activation of biosynthetic pathways leading to production of secondary metabolites. Ghorbanpour and Hadian (2015) noted that application of nanomaterials (MWCNTs) leads to rise in hydrogen peroxide which is correlated with the rise of secondary metabolites such as phenolics, flavonoids, rosmarinic acid, and caffeic acid. Chong et al. (2005) reported that rise in hydrogen peroxide is correlated with anthraquinone biosynthesis in cell suspension culture of *Morinda elliptica*. Similarly, it has been observed that following treatment with silver (Ag) nanoparticles, there is a rise in artemisinin biosynthesis which is accompanied with excessive production of reactive oxygen species. According to Hatami et al. (2018), interaction of nanomaterials with signaling cascades leads to modulation of plant cells and production of reactive oxygen species, cytoplasmic $[\text{Ca}^{2+}]$ concentration, and activation of mitogen-activated protein kinase. However, Hatami et al. (2018) noted that precise details of molecular mechanism of entry of nanomaterials into plant cells are not well understood. Sosan et al. (2016) reported that in *Arabidopsis thaliana*, plasma-bound receptors recognize Ag nanomaterials that trigger Ca^{2+} burst and reactive oxygen species. Similarly, in *Oryza sativa*, it was observed that when Ag nanoparticles are applied to roots, there was a rise in Ca^{2+} concentration and related signaling pathway which was confirmed by proteomic analysis (Mirzajani et al. 2014). Moreover, it is revealed from the analysis of MAPK phosphorylation and transcription factors that transcriptional reprogramming causes activation of secondary metabolism in plant cells (Vasconsuelo and Boland 2007;

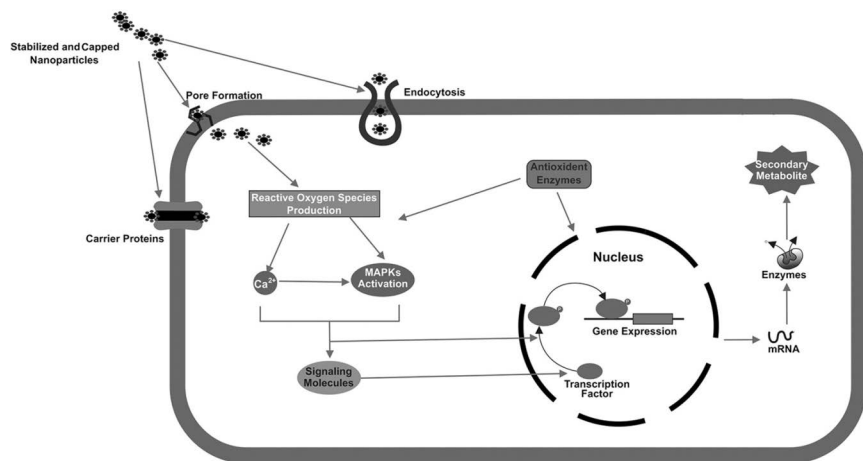


FIGURE 6.1 Probable molecular mechanism of elicitation of plant secondary metabolites through nanoparticle elicitation.

Schluttenhofer and Yuan 2015; Phukan et al. 2016) (Figure 6.1). Although several studies have been conducted on the mechanism of entry of nanomaterials on plant cells, in-depth systematic study would be required to gain complete understanding of molecular mechanism of interplay of nanomaterials with biological surfaces (plant cells).

6.3 BIOSYNTHESIS OF PLANT SECONDARY METABOLITES UPON EXPOSURE TO NANOMATERIALS

6.3.1 ALKALOIDS

Alkaloids are nitrogenous organic molecules that are synthesized from amino acids (tryptophan, tyrosine, phenylalanine, lysine, and arginine) (Pagare et al. 2015). These are also produced in plant cell cultures when exposed to nanomaterials. Ghorbanpour et al. (2015) reported that tropane alkaloids (hyoscyamine and scopolamine) are produced in black henbane (*Hyoscyamus niger* L.) at concentrations of 20, 40, and 80 mg/l when exposed to titanium dioxide (TiO₂) nanoparticles (10–15 nm). They observed that 0.286 g/kg of hyoscyamine was collected by the exposure of 80 mg/l of TiO₂ compared to 0.161 g/kg of hyoscyamine collected by the exposure of TiO₂ BPs (bulk particles), whereas exposure of TiO₂ BPs nanomaterials leads to the maximum yield of scopolamine. Moreover, a higher concentration of biomass and a better synthesis of alkaloids leads to increased yield (2.5 times) of total alkaloids when TiO₂ nanomaterials were applied at 40 mg/l (Ghorbanpour et al. 2015). The rate-limiting enzymes (putrescine N-methyltransferase and hyoscyamine 6 β -hydroxylase) were activated by the exposure of nanomaterials. Similarly, Jamshidi and Ghanati (2017) assessed

taxane production in hazel cells (*Corylus avellana* L.). Suspension cultures of hazel cells were exposed to Ag nanoparticles at a concentration of 0, 2.5, 5, and 10 mg/l during the logarithmic growth phase of cells and were harvested after 1 week. It has been observed that some physiological parameters (membrane stability, growth of cells) were found to be decreasing, while electro-conductivity and total dissolved solids increased accounting for disruption of membrane. Other alkaloids, viz., taxol and baccatin III, were found to be increasing in hazel cells (*C. avellana* L.) when Ag nanoparticles were applied at a concentration of 5 mg/l. Furthermore, the concentration reached to 378% and 163%, respectively, compared to control after 24 hours of treatment. The details of nanoparticle-elicited alkaloid enhancement in *in vitro* plant cell cultures is summarized in Table 6.1.

TABLE 6.1
Effects of Different Types of Nanoparticles Used as Elicitors of Specialized Metabolites in Different Plant Species

Plant Species	Nanomaterial	Elicited Phytochemicals	References
<i>Pelargonium graveolens</i>	Ag-NP	Citronellol, geraniol, citronellyl, formate, isomenthone, linalool, and E-caryophyllene	Mittler et al. (2002)
<i>Bacopa monnieri</i>	Ag-NP	Protein, carbohydrates, phenol	Krishnaraj et al. (2012)
<i>Thymus kotschyianus</i>	Ag-NP	Alpha-terpinyl acetate, thymol	Aghajani et al. (2013)
<i>Momordica charantia</i>	C ₆₀ (OH) ₂₀ -NP	Cucurbitacin, lycopene, charantin, insulin	Kole et al. (2013)
<i>Nicotiana tabacum</i>	Al ₂ O ₃ -NP	Phenolics	Poborilova et al. (2013)
<i>Hypericum perforatum</i>	Fe ₃ O ₄ -NP, ZnO-NP	Hyperoside, hyperforin	Sharafi et al. (2013)
<i>Ricinus communis</i> L.	Ag-NP	Phenolics and antioxidant enzymes	Yasur and Rani (2013)
<i>Artemisia annua</i>	Ag-NP	Artemisin	Zhang et al. (2013)
<i>Rapianus sativus</i>	CeO ₂ -NP	Phenolics, flavonoids	Corral-Diaz et al. (2014)
<i>Calendula officinalis</i>	Ag-NP	Anthocyanin, flavonoids, carotenoids, saponins	Ghanati et al. (2014)
<i>Aloe vera</i>	TiO ₂ -NP and Ag-NP	Aloin	Raei et al. (2014)
<i>Hypericum perforatum</i>	FeO _x -NP, ZnO-NP	Hypericin and hyperforin	Sharafi et al. (2014)
<i>Cicer arietinum</i>	TiO ₂ -NP	Phenolics, gallic, chlorogenic, coumaric acid, tannic acid, and cinnamic acid	AL-oubaidi and Kasid (2015)

(Continued)

TABLE 6.1 (Continued)**Effects of Different Types of Nanoparticles Used as Elicitors of Specialized Metabolites in Different Plant Species**

Plant Species	Nanomaterial	Elicited Phytochemicals	References
<i>Arabidopsis thaliana</i>	Ag-NP	Anthocyanin, flavonoid	García-Sánchez et al. (2015)
<i>Artemisia annua</i>	Co-NP	Artemisinin	Ghasemi et al. (2015)
<i>Satureja khuzestanica</i>	MWCNTs	Phenolics, flavonoid, rosmarinic acid, caffeic acid	Ghorbanpour and Hadian (2015)
<i>Salvia officinalis</i>	TiO ₂ -NP	Camphene, p-cynene, 1,8 cincol, cis-thujene and camphor, phenolics, flavonoids and essential oils	Ghorbanpour et al. (2015)
<i>Pelargonium graveolens</i>	Ag-NP	Citronellol, geraniol, citronellylformate, isomenthanelinalool, and E-caryophyllene	Logeswari et al. (2015)
<i>Glycyrrhiza glabra</i>	ZnO-NP and CuO-NP	Glycyrrhizin, phenolics compounds, flavonoids, and anthocyanins	Oloumi et al. (2015)
<i>Datura metel</i>	Ag-NP	Atropine, atropine	Shakeran et al. (2015)
<i>Capsicum frutescens</i>	Ag-NP	Capsaicin	Bhat and Bhat (2016)
<i>Prunella vulgaris</i> L.	Ag-NP, Au-NP	Phenolics, flavonoid	Fazal et al. (2016)
<i>Verbena bipinnatifida</i>	Cu-NP	Phenolics	Genady et al. (2016)
<i>Corylus avellana</i>	Ag-NP	Taxol	Jamshidi and Ghanati (2017)
<i>Mentha longifolia</i>	Cu-NP	Essential oil contents	Talankova-Sereda et al. (2016)
<i>Cucumis sativus</i>	Cu-NP	Amino acids, ascorbic acid, phenolics	Zhao et al. (2016)
<i>Trigonella foenum-graecum</i>	CuO-NP	Phenolics and flavonoids	Ain et al. (2017)
<i>Amaranthus caudatus</i>	Ag-NP	Phenolics, flavonoids	Azeez et al. (2017)
<i>Solanum lycopersicum</i>	CeO ₂ - NP	Chlorophyll, carotenoid	Hussain et al. (2017)
<i>Corylus avellana</i>	Ag-NP	Taxanes (taxol, baccatin III)	Jamshidi et al. (2016)
<i>Stevia rebaudiana</i>	ZnO-NP	Flavonoids, steviol, glycosides	Javed et al. (2017)
<i>Cichorium intybus</i>	Fe ₃ O ₄ - NP, ZnO-NP	Phenolics, flavonoids	Mohebodini et al. (2017)
<i>Vanilla planifolia</i>	Ag-NP	Phenolics and antioxidant enzymes	Spinoso-Castillo et al. (2017)
<i>Caralluma tuberculata</i>	Ag-NP	Phenolics, flavonoids	Ali et al (2019)
<i>Cucumis anguria</i>	Ag-NP	Flavonols, lignans, hydroxycinnamic and hydroxybazoic, neolignans	Chung et al. (2018a)

(Continued)

TABLE 6.1 (Continued)**Effects of Different Types of Nanoparticles Used as Elicitors of Specialized Metabolites in Different Plant Species**

Plant Species	Nanomaterial	Elicited Phytochemicals	References
<i>Momordica charantia</i>	Ag-NP	Flavanols, hydroxycinnamic	Chung et al. (2018b)
<i>Withania somnifera</i>	CuO-NP	Phenolics, flavonoids, tannin	Singh et al. (2018)
<i>Brassica rapa</i>	CuO-NP	Gluconasturitin, glucobrassicin, 4-methoxygluco brassicin, neoglucobrassicin, 4-hydroxyglucobrassicidin, glucoallysin, glucobrassicinapin, sinigrin, progoitrin, gluconags and flavonoids, hydroxybenzoic and hydroxycinnamic acid	Chung et al. (2018c)
<i>Stevia rebaudiana</i>	Cu-NP, Au-NP	Phenolics, flavonoids	Ghazal et al. (2018)
<i>Stevia rebaudiana</i>	Ag-NP	Stevioside	Golkar et al. (2019)
<i>Oryza sativa</i>	Ag-NP	Carotenoids	Gupta et al. (2018)
<i>Stevia rebaudiana</i>	ZnO-NP, CuO-NP	Phenolics, flavonoids	Javed et al. (2018)
<i>Echinacea purpurea</i>	ZnO-NP	Flavonoids	Karimi et al. (2018)
<i>Lilium ledebourii</i>	ZnO-NP	Flavonoids	Karimi et al. (2018)
<i>Cichorium intybus</i>	CuO-NP	Phenolics, flavonoids	Laishram et al. (2018)
<i>Hyoscyamus reticulatus</i> L.	Fe ₃ O ₄ -NP	Hyoscyamine, scopolamine	Moharrami et al. (2018)
<i>Atropa belladonna</i>	Mn ₂ O ₃ -NP	Alkaloids, phenolics, flavonoids	Tian et al. (2018)
<i>Trigonella foenum-graecum</i> L.	Ag-NP	Diosgenin	Anjum et al. (2019)
<i>Salvia mirzayanii</i>	MWCNT	Phenolics	Chegini et al. (2017)
<i>Gymnema sylvestre</i>	CuO-NP	Phenolics, flavonoids, gymnemic acid	Chung et al. (2019a)
<i>Brassica rapa</i>	CuO-NP	ROS, hydrogen peroxide, glucusin, proline, anthocyanin	Chung et al. (2019b)
<i>Prunella vulgaris</i> L.	Ag-NP, Au-NP	Phenolics, flavonoids	Fazal et al. (2019)
<i>Dracocephalum kotschy</i>	SiO ₂ -NP	Rosmarinoc acid, xanthomicrol, isokaempferide, and cirsimarifin	Nourozi et al. (2019)
<i>Hyoscyamus reticulatus</i> L.	ZnO-NP	Tropane, alkaloids, phenolics	Sanchez-Munoz et al. (2019)
<i>Glycyrrhiza glabra</i>	Ag-NP	Quercetin, glycyrrhizin	Tahoori et al. (2019)

6.3.2 PHENYLPROPANOIDS AND TERPENOIDS

Phenylpropanoids are one of the complex groups of chemicals which are widely distributed in plants and are derived from cinnamic acid and coumaric acid via the shikimic acid pathway. Terpenoids belong to numerous and structurally diverse kind of natural chemicals consisting of isoprene molecules synthesized by plants (Tholl 2015). Yarizade and Hosseini (2015) treated hairy root culture of *Artemisia vulgaris* with nanocobalt and nanozinc (0, 0.25, 0.5, and 1 mg/l) and checked the expression profile of genes, viz., amorphadiene synthase (ADS), double-bond reductase 2 (DBR2), aldehyde dehydrogenase 1 (ALDH1), and squalene synthase genes (SQS) at 8, 24, 48, and 72 hours of interval. They observed that application of cobalt nanomaterials (0.25 mg/l) and nanozinc (1.0 mg/l) leads to increment of expression profile of genes. Production of artemisinin using hairy root culture mediated by nanomaterials (nanozinc and nanocobalt oxide) was suggested. Owing to simultaneous increase in ADS regulation, nanocobalt has proved to be a better elicitor than nanozinc (Yarizade and Hosseini 2015). Baldi and Dixit (2008) studied the effect of metal ions Co^{2+} and Zn^{2+} on suspension culture of *Artemisia annua* L. and found a slight increase in the content of artemisinin. Similarly, Bahreini et al. (2015) analyzed the constituents of *in vitro*-grown fennel plantlets which were elicited by TiO_2 and SiO_2 and compared with untreated plantlets. They found in untreated plants, a synthesis of four chemicals, viz., anethole, fenchone, limonene, and decane, and in plants treated with TiO_2 , they found dodecane, phytol, and phenol and along with octane, which was most available. Treating plants with SiO_2 -based nanomaterials led to the production of benzoic acid, jasmonic acid, hexadecanoic acid, pyrrolidinone, phytol, and benzoic acid (Bahreini et al. 2015). Ghorbanpour (2015) used TiO_2 nanomaterials and found that on exposure of these nanomaterials on *Salvia officinalis*, there was an increment in oil content and yield. At 200 mg/l of TiO_2 , there was an increment of oil content by 1.75 and yield by 2.74 than untreated plants. It was found that on exposure of 200 mg/l TiO_2 , the concentrations of *cis*-thujene and 1,8-cineol reached highest. Similarly, Zhang et al. (2013) observed that application of 900 mg/l Ag nanomaterials on *A. annua* hairy root cultures led to an increase in artemisinin content after 3 days. Ghorbanpour and Hatami (2015) studied the cumulative effects of varied concentrations of Ag nanoparticles (5–35 nm) (20, 40, and 80 mg/l) and thidiazuron (TDZ) (0, 50, 75, and 100 μM) and found that they led to accumulation of important oil constituents in Geranium (*Pelargonium graveolens*) plants. Specifically, contents of essential oils like citronellol (C) and geraniol (G) are increased by the combined application of 80 mg/l of Ag nanomaterials and 100 μM of TDZ. Furthermore, it was seen that combination of 40 mg/l Ag nanomaterials and 75 μM of TDZ leads to C/G ratio equal to one which showed positive interactions between Ag nanomaterials and TDZ. They opined that accumulation of secondary metabolites resulted from the generation of hydrogen peroxide which is formed by the application of Ag nanomaterials and/or TDZ in a dose-dependent manner. They concluded that co-exposure of Ag nanomaterials with plant growth regulator (e.g. TDZ) at appropriate concentrations could be a

promising technique to elicit phytochemicals from *in vitro* plant cell cultures. The details of nanoparticle-elicited phenylpropanoids and terpenoids enhancement in *in vitro* plant cell cultures are summarized in Table 6.1.

6.3.3 FLAVONOIDS AND PHENOLICS

Phenolics and flavonoids are a group of important compounds synthesized through the shikimate phenylpropanoids-flavonoids pathway in plants. They exhibit reactive oxygen scavenging activities and protective roles against oxidative damages (Osawa 1994). Raei et al. (2014) studied the effects of nanomaterials (Ag, TiO₂) on the cell suspension culture of *Aloe vera*. *A. vera* has anthraquinones which show antimicrobial activities and have other pharmacologically important properties (Reynolds 2004; Hasanuzzaman 2008). They added Ag nanoparticles at 6, 24, 48, 72, and 168 hours of intervals and collected induced calli. It was observed that after adding Ag nanoparticles, anthraquinones were collected from induced calli in 48 hours, but the production was reduced with time. They opined that reduction might have happened because of the excessive production of anthraquinones and feedback of anthraquinones on gene expression. In experiments done with titanium dioxide (TiO₂), anthraquinones were produced in 48 hours following elicitation, but declined with time by 8.8%. The reason for the decline was attributed to the toxic effect of nano-TiO₂ in the culture medium or on gene expression. Similarly, *Bacopa monnieri* (L.) (Brahmi) was utilized to study the effect of Ag nanomaterials on metabolism by Krishnaraj et al. (2012). They found that after treatment with Ag nanomaterials, total phenol content was found to be improved in *B. monnieri* (Linn.) (Brahmi) grown in hydroponic solution. They inferred that increased phenol content was maybe due to the stress imposed by Ag nanoparticles on the growth and metabolism of *B. monnieri*. Sharafi et al. (2013) used iron- and zinc-nanooxides as elicitors for the production of hypericin and hyperforin in cell cultures of *H. perforatum*. Hypericin and hyperforin are naphthodianthrone and prenylated acylphloroglucinols, respectively, which are used to treat depression (Deltito and Beyer 1998; Dias et al. 1998). Different concentrations of nanomaterials (zinc- and iron-nanooxides) (0, 50, 100 and 150 ppb) were added in the cell suspension cultures, and the samples were examined after 72 hours. They observed that hypericin and hyperforin productions are increased by the zinc- and iron-nanooxides at the 100 ppb concentration in cell suspension culture of *H. perforatum*. When zinc oxide nanomaterials were added to the cultures, the contents of hypericin and hyperforin increased to their maximum values of 7.87 and 217.45 µg/g dry weight, respectively. Compared with control, these were 3- and 13-folds higher, respectively. Cultures treated with nanomaterials (iron oxide) yielded 11.18 µg/g dry weight of hypericin and 195.62 µg/g dry weight of hyperforin. It has been observed that hyperforin production was more than hypericin by the treatment of zinc- and iron oxide nanomaterials. The study showed that secondary metabolite production in *in vitro* conditions is feasible by elicitation of nanomaterials. AL-Oubaidi and Kasid (2015) studied nanomaterials-mediated elicitation of phenolic and flavonoid productions in gram (*Cicer arietinum* L.).

Nanomaterials (TiO_2) were added in different concentrations (0.5, 1.5, 3, 4.5, and 6 mg/l). It was observed that callus induced from the embryo of gram yielded 4.5 and 6.0 mg/l, and the observed increment as compared by untreated plant was confirmed quantitatively using high-performance liquid chromatography (HPLC). Similarly, Khan et al. (2016) evaluated the effect of nanomaterials (Ag, Au, Cu, AgCu (1:3), AgCu (3:1), AuCu (1:3), AuCu (3:1), AgAu (1:3), AgAu (3:1)) on the total phenolic and flavonoid contents in milk thistle plant. Seeds of this plant were soaked in nanomaterial suspensions for 2 hours and were allowed to grow under *in vitro* conditions for six weeks. On weekly basis, the samples were collected for estimation of phenolic and flavonoid contents. It was observed after 21 days that in the seeds soaked in bimetallic alloy, phenolics and flavonoids contents increased, whereas in the seeds soaked in monometallic nanomaterials, phenolic and flavonoid contents reduced. After completion of 28 days, total phenolics and flavonoids reached the maximum accumulation by Au and Cu nanomaterials. It was concluded from the study that monometallic nanomaterials exerted maximum effect on secondary metabolite production. Furthermore, factors like composition of nanomaterials, size, and surface area played key roles either singly or together in elicitation of secondary metabolites. Similarly, Oloumi et al. (2015) studied the effect of nanomaterials (CuO and ZnO) in the concentrations of 1 and 10 μM , respectively, in the agar growth medium containing Hoagland nutrient solution on *Glycyrrhiza glabra* seedlings. They found that after the treatment, glycyrrhizin and phenolics contents increased. Elicitation of flavonoids and phenolics in *in vitro* plant cell cultures by nanomaterials is mentioned in Table 6.1.

6.4 CONCLUSION AND FUTURE PERSPECTIVE

Nanomaterials have shown potential in elicitation of secondary metabolites from plant cell cultures in recent years. Studies conducted by authors revealed complex interactions between nanomaterials and plant cells owing to the different factors associated with nanomaterials (dimension, optimum concentration, entry time, and route) and plants (species difference, age, culture conditions) (Basiuk et al. 2011; Dey and Das 2013; Hatami et al. 2018; Verma et al. 2018). A more thorough analysis would be required using classical and omics-based approaches to gain a comprehensive understanding of the mechanism of elicitation of secondary metabolites mediated by nanomaterials. Classical techniques based on *in vitro* plant cell cultures (cell suspension, callus, and embryo cultures) and omics-based approaches like 2D gel electrophoresis-based proteomics, EST databases, and cDNA-amplified fragment length polymorphism (AFLP) technology can also be utilized for understanding the mechanism of elicitation of secondary metabolites (Goossens et al. 2003; Bhatia and Bera 2015). Addition of nanomaterials onto culture media (semi-solid or liquid) may help to form desired *in vitro* morphogenic cultures (callus or embryos) which would secrete secondary metabolites. Subsequently, specific proteins employing 2D gel electrophoresis can be isolated which may shed light on the mechanism of elicitation of secondary metabolites. Similarly, an annotated EST database of collection of unique genes from plant cell cultures involved in elicitation of secondary metabolites can be created and

DNA chips can be prepared which would be useful for expression analysis of selected genes playing a role in elicitation of secondary metabolism. Techniques like cDNA-amplified fragment length polymorphism (AFLP) would be useful in identifying the genes of secondary metabolites as it does not require prior sequence information and provides expression profiles in quantitative mode (Goossens et al. 2003). Furthermore, unexplored nanomaterials like Fe_2O_3 , CoO , NiO , Nd_2O_3 , fullerene, fullerenes, graphene, and carbon dots can be tried to figure out their roles in elicitation of secondary metabolites (Anjum et al. 2019).

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7 Impact of Nanomaterials on Health and Environment

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7.1 BASIC CONCEPTS OF NANOTECHNOLOGY

Nanotechnology means the manipulation and the study of matter at the nanometer scale to generate useful devices and materials. A scientist employing on “life sciences interface” might tell that it is determining methods of connecting antibodies to the magnetic nanoscale particles to invent treatments of cancer [1,2]. A researcher working on molecular electronics can generate self-assemblies of nanoscale particles to develop tiny active parts in electronic circuits that are much smaller than a transistor on IV chip of Pentium. Some nanotechnologists are discovering ways to develop small robots whose parts are at the molecular level (nanorobots). There are four groups of nanotechnologies including incremental, radical, evolutionary, bottom-up/top-down nanotechnology. First, we explain incremental nanotechnology [3,4]. All chunks, even solid metal substances, having a grain-like structure allow developing better-performing nanomaterials (NMs). This means stronger metals, interruptions of nanoparticles (NPs) with modified properties, magnetic films with large magnetization, and so on. In fact, some features of incremental nanotechnology were already examined in the past [2,5]. While incremental nanotechnology is stated in assembling a huge number of small particles to generate functional matter, evolutionary nanotechnology means to produce nanoscale particles that can individually operate as a useful device. These may be assembled in huge numbers to produce a macroscopic thing to form a useful material, but functionality is activated in each nanoparticle. Such nanoscale particles are more complicated than those who worked in incremental nanotechnology [6,7]. The very far-reaching form of nanotechnology, defined as radical nanotechnology proposed by Richard Feynman, is the building of devices whose mechanical parts are at the level of molecules. Radical nanotechnology has divided into two different types, namely, (i) molecular engineering, in which macroscopic devices and structures are formed by assembling their fundamental atoms, and (ii) nanobots or nanorobots, which are invisibly tiny mobile devices [8,9].

Lastly, the worth-mentioning method for classifying nanotechnology is described as bottom-up and top-down nanotechnology. In bottom-up technique, basic building blocks (molecular device component, NPs, etc.) can be generated naturally and then brought together to form the required device and material [10]. The top-down approach is similar to conventional manufacturing using millers and lathes to produce a structure out of a crystalline solid block. The advanced tools of nanotechnology, though, are able to produce structures with a few nanometers size; therefore, the size of the components produced by top-down technique is not very dissimilar from the basic block of the bottom-up technique. The flexibility of tools in the top-down technique, specifically focused ion beam systems (FIBs), is more improved by their capability to deposit NM and generate nanoscale structures as well as to eliminate them [11].

The two (top-down and bottom-up) approaches are complementary, and advanced research increases the amalgam of the two approaches. For example, if an approach wants to calculate the magnetic and electrical properties of a single

nanoparticle, the accuracy of an advanced top-down approach can help in the formation of electrodes that can connect to it. Generally, both approaches can be separately applied to each of the radical, incremental, and evolutionary categories of nanotechnology [12,13].

Nanoscale materials are foundations of nanotechnology and nanoscience. Nanostructure technology and science is an interdisciplinary and broad area of developing activity and research which has been rising explosively in the whole world from past several years. NMs have the potential for developing the ways in which products and materials can be produced and also change the nature and range of functionalities [14,15]. Human hair diameter is approximately 100,000 times greater than a nanometer. Materials at nanoscale can be one-dimensional (coating), two-dimensional (nanowires), and three-dimensional (NPs). NMs can accrue in aggregated, fused, agglomerated, or single forms with irregular, spherical, and tubular shapes. Common shapes of nanoscale materials include fullerenes, dendrimers, nanotubes, and quantum dots. Nanoscale materials are very important because, at this level, unique electrical, optical, magnetic, and many other emerging properties. These developing properties have the potential for large influences in medicine, electronics, and many other fields [16]. Some nanoscale materials are found naturally, but of specific interest, are manufactured in the laboratories [17,18].

Manufactured nanoscale materials are resources structured at the molecular size to take benefit of their novel properties and small size. There are two major reasons for enhanced properties, i.e. novel quantum effects and relative increased surface area. Materials at nanolevel have increased surface area to volume ratio as compared to conventional bulk materials, which can increase their effect of strength and chemical reactivity. Similarly, nanolevel quantum effects have much importance in finding materials' properties, which leads to identification of new magnetic, optical, and electrical behaviors [19,20]. A variety of commercial nano-products are available in the market, including wrinkle-free and stain-resistant textiles, paints, varnishes, and sun block creams [21].

7.1.1 SYNTHESIS METHODS

There are various methods for synthesizing NMs. Atoms in NMs can organize by ways depending on equilibrium thermodynamics by self-assembly and self-organization. By employing controlled synthesized processes, manufactured materials can be organized into valuable shapes, and the materials can be used to some specific applications (Figure 7.1) [22,23]. Now, we will discuss the basic ideas of few synthesis methods.

The sol-gel method involves the inorganic network evolution through the generation of colloidal (sol) suspension and gelation of the colloidal suspension to make a network in a steady aqueous phase (gel). The precursors for forming these NPs normally consist of a metalloid or metal element confined by different reactive elements. The synthesized material is processed to make a dispersible oxide colloidal suspension in contact with a dilute acid or water. The liquid is removed

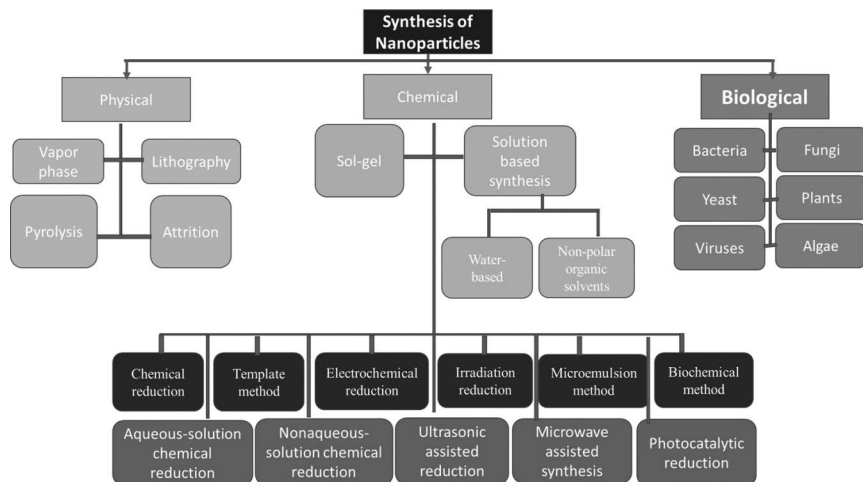


FIGURE 7.1 Distribution of synthesis protocols for NPs (Arshad, 2017) [26].

from the yield of sol–gel, and the transition of sol/gel controls the shape and size of the particles. The oxides are produced by the calcination of gel [24,25].

Mechanical grinding method is an ordinary example of “top-down” process for the synthesis of NPs, where NMs are formed by the structural breakdown of grained coarser structures as a result of plastic deformations. This method has become popular to form crystalline NMs due to its relatively low-price equipment, simplicity, and capability to form all the types of materials [27]. Its main advantage is that the possibility of conveniently scaling up the material tonnage quantities for different applications instead of cluster aggregation [28]. However, there are two main problems associated with the method:

1. Contamination from the atmosphere or milling media.
2. The power product consolidated without increasing the nanocrystalline structure.

The laser ablation process has been mostly used for synthesizing particulate films and NPs. A laser beam is used as a primary source for excitation of ablation for producing nanoclusters directly from different solid samples. The small-scale dimensions of the NPs and the achievability to make thin films form this process quite efficient method for preparation of coating, ceramic particles, and also a source of ablation for analytical uses such as joining to emission spectroscopy of induced coupled plasma (ICP) [29,30].

The hydrothermal synthesis works on a single-phase heterogeneous reaction in liquid-like media at elevated pressures and temperatures to form hydrous ceramic and crystallized materials from solutions. This synthesis method provides a direct route for formation of oxides, sulfides, and different composite materials. The technical aspects of hydrothermal reaction carry a model of aqueous nucleation.

The properties of synthesized materials depend on chemical kinetics, thermodynamic properties, and chemical equilibrium of the liquid system under the hydrothermal conditions [31,32].

7.2 TOXICITY OF NMs

Nanotoxicology relates to the phenomenon of toxicity in NMs. It can be induced due to different reasons such as manufacturing process, naturally occurring process, and combustion process. The consequential differences in chemical and physical properties of NMs as compared to bulk materials are widely recognized. Nowadays, the exposure of NMs to humans has increased, which highlights their significance, and this can produce long-term impacts on human health. Due to their tiny sizes, NMs can enter into the human body through skin penetration, ingestion, inhalation, and connection with intracellular macromolecules and structures for a long interval of time [33].

There are several mechanisms that can produce toxicity in NMs, but many in vivo and intracellular toxicities of NMs emerge from the generation of reactive oxygen species (ROS) (Figure 7.2) [34,35]. For modulation of different cellular events, such as protein redox regulation, gene expression, proliferative response, and signal transduction, the role of moderate levels of ROS is very important. However, the high level of ROS can indicate the oxidative stress which can cause damages to cells by altering proteins, interfering with the function of signaling, disrupting DNA, modulating genes, and peroxidizing lipids and then ending up in pulmonary, cardiovascular, neurological degenerations, renal diseases, and cancers [36].

ROS steals the electrons from the membrane of the lipid cells, which results in the reduction of physiological function, and finally, cell death. For example, TiO_2 NPs can induce an oxidative stress which results in a pulmonary inflammatory

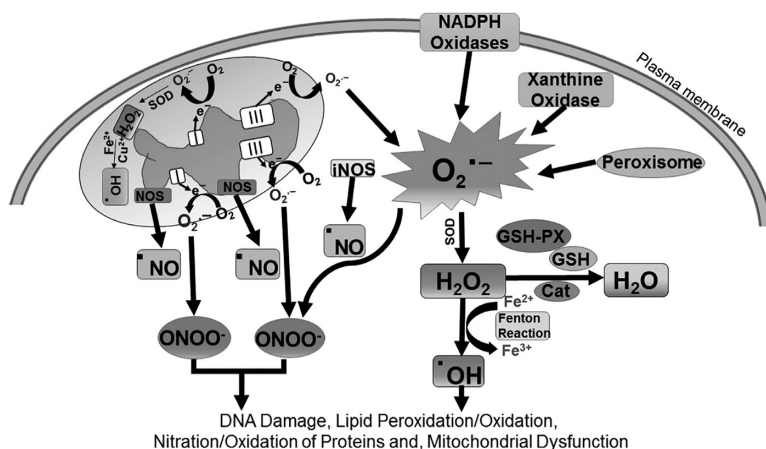


FIGURE 7.2 Processes arose due to generation of ROS (Kaushal et al., 2019) [37].

response in rats. The carbon-based materials can be correlated with oxidative stress indication, which is present in rat and fish brain cells. Additionally, the toxicity caused by ROS can be highly pronounced inside the central nervous system because of the presence of a large amount of unsaturated fatty acids [35]. There exist three stages in which NMs produce oxidative stress, which affects the signaling of cells. At the first stage, the transcription related to defense genes enhances. At the second stage, the inflammation signaling of oxidative stress is activated. In the last step, the necrosis and apoptotic pathways are activated. If the signaling pathways are changed in the cell, it results in carcinogenic effects [35].

Toxicology of NMs can be studied by *in vitro*, *in vivo*, and *in silico* approaches. At the early stages, the *in vitro* approach has a number of advantages due to its achievable high reproducibility and can provide information at cellular and tissue levels. However, this method gave little information on the cause and mechanism of cellular toxicity [38]. The reliability of *in vitro* method can be verified by studying the toxicity in rats using different NMs. The comparison of *in vitro* and *in vivo* measurements provides correlation between toxicity at the cellular level. Hence, *in vitro* systems are most convenient for the identification of some specific effects induced by NMs. This method can be useful in indicating the toxicity and studying the different induced mechanisms [39].

There are few aspects that cannot be studied by *in vitro* system, e.g. *in vitro* toxicity provides short-term toxic effects; therefore, to study longer toxicity effect and in actual microenvironment, the animal model is preferred. *In vivo* experiments are expensive, are time-consuming, and have ethical issues. However, such experiments can give detailed information about pulmonary, carcinogenicity, and gastrointestinal and dermal toxicities, which are related to NMs injected through several exposure routes. The toxicity of NMs can also be predicted by *in silico* methods which replace few expensive materials and time-consuming approaches [40,41]. Quantitative structure–activity relationship can be used for identification of biological and physicochemical properties of molecules. The *in silico* toxicology technique is cost-effective as well as time-saving as compared to bioassays that are used to identify the toxic effects of NMs. Due to excess use of NMs, the data of *in vivo* toxicity are continuously required to determine the possibilities of risks that are associated with new materials. Humans can be exposed to NMs through inhalation, orally, intraperitoneally, transdermally, and intravenously [42].

NMs can spread out in different organs where there is a possibility of remaining in the same structure or might be metabolized or modified. There is a possibility of NPs to enter into different tissues and remain in the cells for a long period of time [43]. Several experiments have focused on NPs with known toxicity characteristics, but these experiments are insufficient to estimate the biological interactions with NPs. For instance, the absorption effect of quantum dots on porcine skin is commonly related to surface coating chemistry. Such studies offer the significance of physical properties and the adsorption behavior of the exposure route of nanostructures. Potential toxicity and NM activity are highly required to analyze the pharmacokinetics [44].

The characteristics of NMs including size, shape, chemical composition, agglomeration state, and surface characteristics such as surface area, surface charge, surface energy, surface coating, and surface morphology, influence their toxicological potential and biological behavior. In the field of nanotoxicology, the particle size is one of the crucial parameters. Surface area increases exponentially with decrease in size. Thus, the surface of NMs becomes more reactive for biological components as well as for itself [45,46].

7.3 EXPOSURE ROUTES OF NMs

To study the hazards of nanoscale materials, it is important to understand possible exposure routes of different types of nanoscale materials. The exposure of NPs to humans and the environment can be explained through various mechanisms. First, there are occupational exposures to workers (including technicians, scientists, and engineers) during the commercial production and research-scale synthesis of nanoparticle-based products. Second, consumers have exposed to such nanoscale materials during application and usage, and it may lead to toxic and harmful effects. Primarily, there are four routes of NM exposure to human body, namely, inhalation, dermal, ingestion, and injection (Figure 7.3) [47].

7.3.1 INHALATION

The human lungs have very large surface area, i.e. 50–75 m², for gas exchange of carbon dioxide and oxygen. The breathing air holds a variety of species such as dust particles, germs, or other impurities. The human lungs have particular

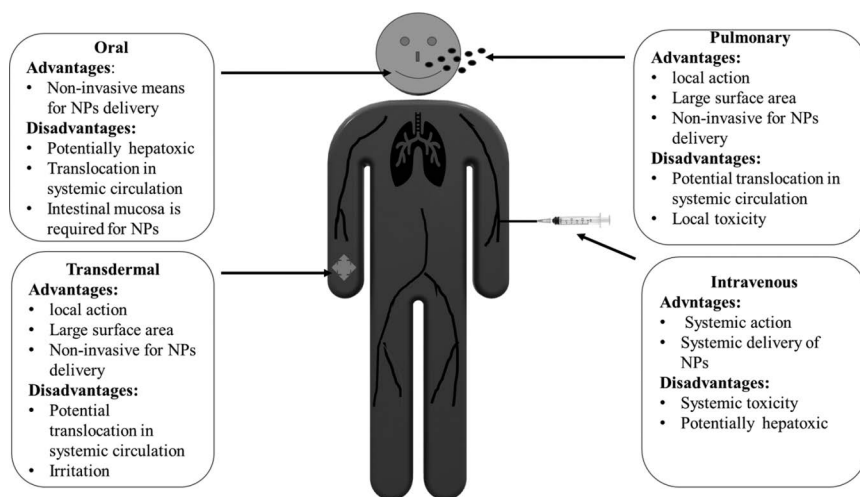


FIGURE 7.3 Different exposure routes of NPs and their advantages and disadvantages (Yildirim et al., 2011) [48].

clearance mechanisms to control such contaminations. The clearance mechanisms of the human body contain two different principles: ciliated mucociliary and alveolar macrophages of upper airways [49]. The particles move in the various areas of the human lungs where particles reach in the alveolar region by macrophages, which then transfer upward to the bronchi and bronchioles. Large particles are moved upward by mucociliary clearance, also named the mucociliary escalator. Later, this mucus, consisting of various substances, is removed from different regions of the lungs by swallowing, coughing, or spitting [50].

The particulate size is significant for deposition in the respiratory tract and possible transfer in the blood. In this relation, the definitions of the dust by WHO differentiate between thoracic, inhalable, and respirable dust. The fraction of inhalable dust can spread the upper airways like throat, mouth, and nose; the fraction of thoracic is smaller and can enter the upper airways of the human lungs. The fraction of respirable dust consists of micro-level particles which can penetrate into gas exchange region, alveoli, and cross the cell layers to enter in the bloodstream. Since NPs are smaller than the fraction of particulate matter, they cross the air–blood bar in a similar way. However, the commonly applied dose will be transferred via macrophages and elated out of the human lungs [51,52]. The possibility of gold NPs to cross the blood–air bar is dependent on the surface area. The gold NPs (1.4 nm) with the largest surface area were stated to translocate the maximum. Both accumulation and translocation depend on the characteristics of particles such as surface charge and surface area [52,53].

It is observed that a small portion of the overall inhaled dose influences the bloodstream and internalizes in organs such as kidney, spleen, and liver. NPs may induce cardiovascular, respiratory, and inflammatory diseases [54,55]. The inhaled NPs translocate into the vascular inflammation sites by systemic circulation and provide a link between cardiovascular disease and NPs present in the environment, necessary to understand consequences of synthesized NPs [56].

Many observations [57] have explained that inhalation of a large number of engineered NPs or metal oxides, which would be just a fraction of granular bio-persistent particles, could cause inflammation to the lungs [58]. Furthermore, the geometric structure/shape of NPs is also a critical factor that causes toxicity to the lungs. The structural resemblances between manufactured nanofibers and mineral fibers, including nanowires, nanotubes, and nanorods, have been stated as a concern to have similar properties as asbestos fibers in controlled conditions. It has also been observed that only long asbestos fiber and thin and long nanofibers are responsible for extensive fibrosis and long-term inflammation in lung tissues [59].

7.3.2 DERMAL

The dermal exposure of hazardous materials continues to rise [60]. Moreover, detrimental effects within the skin are increasing through skin exposure of NMs, or alternatively, the material can be absorbed via skin, circulate through the bloodstream and cause universal effects [61]. The human skin exposure to nanoscale materials can occur through unintentional and intentional means. The intentional

exposure of nanoscale materials may result from application of cosmetic products such as sunscreens, creams, and lotions having NPs of titanium dioxide and zinc oxide. These NPs are assumed to be activity improvers for cosmetics. The exposure of skin to synthesized nanoscale materials can also happen during treatments with drugs and other topical creams [61]. The unintentional exposure to the skin occurs via directly engineered NPs during disposal, manufacturing, and combustion of used NP-based products. Other sources of unintentional exposure of NPs could be natural gas, tailpipe emission, NPs generation during skin waxing, natural powder/gas equipment, plants of oil-fired power, welding fume emission, and emission from coal [62].

There are two different methods of nanoparticle diffusion into the skin: penetration through hair cavities or intercellular trans-epidermal process (Figure 7.4). Absorption of NPs can occur through various routes including sweat ducts, hair follicles, and transcellular cell paths [63]. The concerns about the diffusion of NPs in the skin and resulting toxic effects are debatable issues among scientists and researchers. These concerns include accumulation of toxicity in the skin, photoactivation of NMs that exist in the skin, cytotoxicity of the skin, and metabolisms with toxicity potential. The skin of humans is an actual barrier toward toxic chemicals and NMs; however, the presence of sweat glands and hair follicles makes this barrier susceptible via simplifying diffusion of small-sized NPs [64]. Fewer nanoscale materials are detected over viable skin, while large diffusion happens through follicles of hairs when the skin's protective layer is wounded,

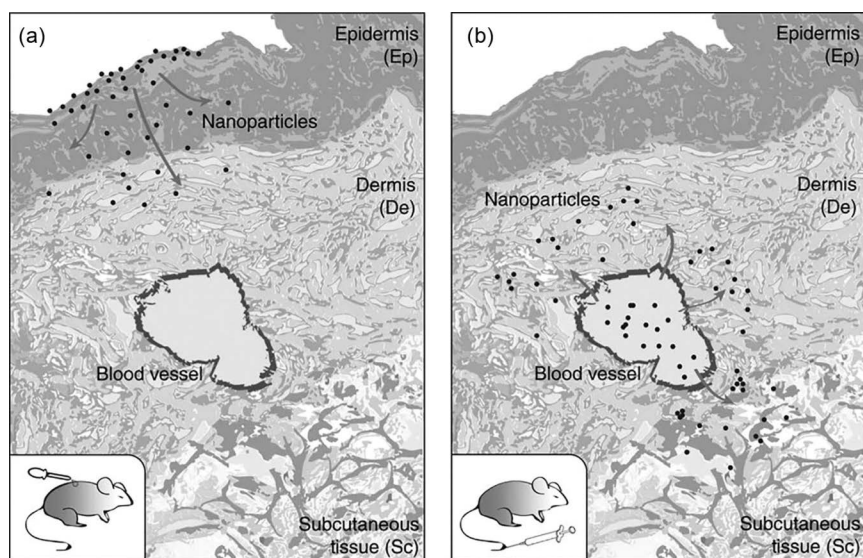


FIGURE 7.4 Entry routes of NPs in mice injected through (a) topical dermal application and (b) tail-vein. NPs spread in dermis and epidermis when applied topically. After administration, NPs only reached hypodermis (subcutaneous tissues). Reprinted with permission from (Sykes, E.A., et al., 2014) [68].

damaged, or removed. The surface coating of titanium dioxide NMs can indirectly harm the skin, which results in nanoparticle diffusion into the skin. The use of NPs for treating damaged skin and wounds can accelerate the diffusion [65].

Many products often consist of substances that can improve the diffusion of NMs into the skin. When marked with keratinocytes in tissue culture, carbon nanotubes lead to the creation of ROS, oxidative phosphorylation, and mitochondrial dysfunction. Nanoscale materials can also persuade a toxic effect inside the skin dominating to inflammation. NMs can unmask epitopes and denature proteins; for example, soot NPs from diesel consume antigen via dendritic cells [66]. It has been analyzed that synthesized NPs such as multi- or single-walled carbon nanotubes, surface coating, and quantum dots can have lethal effects on epidermal keratinocytes and fibroblasts and are able to change their protein or gene expression [67].

7.3.3 INGESTION

Improvement of drug administration based on synthesized NPs is one of the great promises of nanotechnology, and oral ingestion is possible to be an important tract route. Numerous studies have already shown that NPs are competently absorbed via the gastrointestinal tract and then translocated through the mucosal tissues into the circulatory and lymphatic systems (Figure 7.5) [69]. However, the risk from accidental exposure of NPs is not very clear.

The gastrointestinal tract is a system responsible for consuming and digesting foodstuffs, absorbing nutrients, and expelling the waste. Gastrointestinal exposure can occur through direct ingestion of vegetables, drugs, water, and foodstuffs with adsorbed NPs, where NPs penetrate into lymphatic cell tissues [70].

A large number of factors are involved in monitoring the absorption of NPs in gastrointestinal tract, including particle size, ligand type, surface charge, geometry, and potential attachment to the ligand (Figure 7.6). In addition, NPs cleared from the respiratory tract through mucociliary escalators (swallow and coughs) may be ingested into the gastrointestinal tract. So, gastrointestinal tract is a vital

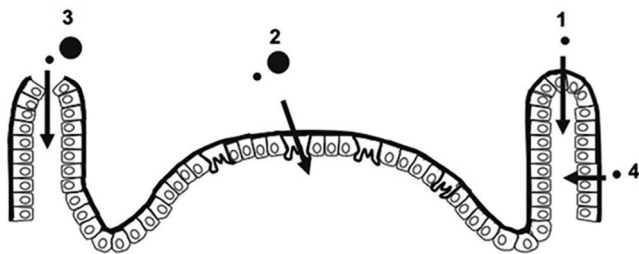


FIGURE 7.5 NPs crossing the gastrointestinal track: (1) NPs in the size range from <50 to 100 nm crossed epithelial cells through endocytosis, (2) microparticles crossed M-cells through transcytosis, (3) passage of nano- and microparticles through villous gaps, and (4) uptake of small NPs through paracellular route. Reprinted with permission from (Powell, J.J., et al., 2010) [71].

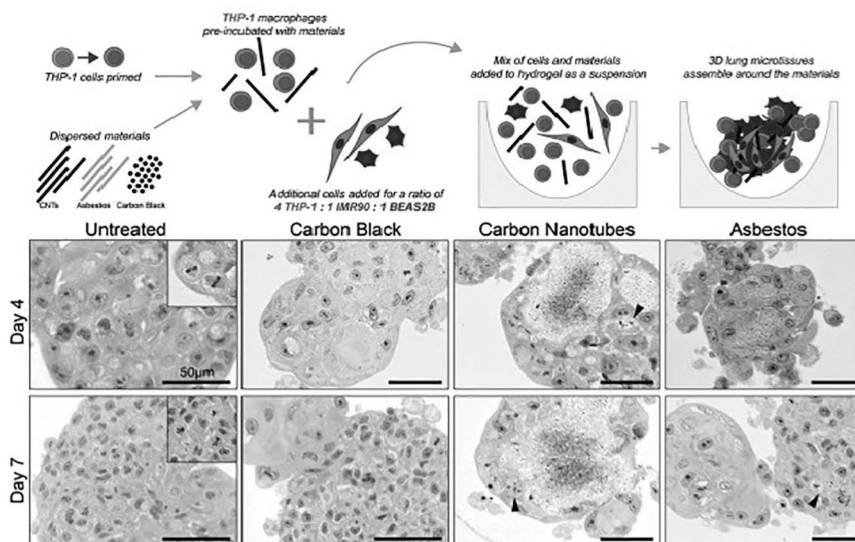


FIGURE 7.6 Morphological changes induced in human lung 3D microtissues upon exposure to NMs (Kabadi et al., 2019) [73].

target for NPs exposure [71]. NPs disposed into water bodies can accumulate in sea foods, and these poisoned foods can act as a source of ingestion. Toxicity induced by the ingested NMs of titanium dioxide can harm the gland cells of the digestive membrane via an oxidative strain mechanism [72].

7.4 OVERVIEW OF RISK ASSESSMENT

Risk assessment describes the level of risk, usually relative ranking or score. The purpose of risk assessment is to provide promising/favorable information to find alternatives. Risk assessment is classified into the following four categories:

- Hazard assessment.
- Dose-response assessment.
- Environmental exposure assessment.
- Risk characterization.

7.4.1 HAZARD ASSESSMENT

Hazard identification requires scientific results to describe the properties of substances or chemicals and their possible adverse health effects on terrestrial, human, and aquatic lives. Hazardous materials, when come in contact or enter the body, interact with cells systematically or locally and cause tissue damage [74]. Later, the negative impact of NMs on the environment and human health receives considerable attention. However, changes have taken place in the past few years, and numerous scientific researches have indicated that exposure to certain NPs can

cause harmful effects on various organs of experimental animals. Several studies have been conducted to assess the ecotoxicity and toxicity of NPs. Schneider et al. [75] studied the toxicity of NPs with different chemical compositions. The studies included cytotoxicity, DNA damage, microbial testing, mammalian toxicity, and ecotoxicity. Several scientists, governmental and non-governmental institutions have examined safety, environmental, and health issues of NPs [76].

i. Carbon Nanotubes

Carbon nanotubes attracted a lot of attention due to their unique physical, fiber-like structural and chemical characteristics, and they are expected to be widely used in various fields, for example, medicine and electronics. Moreover, concerns are raised about the safety issues of the carbon nanotubes because of its insolubility in the lungs. Lam and coworkers reported that single-walled carbon nanotubes can cause lesions and interstitial inflammation in rats between 7 and 90 days [77]. Gomes et al. used carbon materials such as activated carbon, carbon xerogels, and multi-walled nanotubes for the synthesis of platinum catalysts, which were used for aqueous aniline solutions treatment by catalytic air wet oxidation. The synthesized catalysts and materials were analyzed through different tools including scanning electron microscopy and transmission electron microscopy. All catalysts offered a higher rate of aniline and total organic carbon removal activity toward the environmental applications [78].

ii. C60 Fullerenes

In the past few decades, the scientific community is highly interested in fullerenes due to their unique characteristics, e.g., endohydrolytic and superconductivity, and promising applications in biomedical, electronic, and medical fields. However, the safety of C60-based materials is a great concern due to their potential risks to health and the environment. Although the solubility of C60 is poor in water and stable for years, several techniques have been used for the preparation of dispersible (nC60) colloidal polymers in aqueous solutions [79]. Sayes et al. modified C60 fullerenes to minimize the lipophilicity in reduced toxicity [80].

iii. Quantum Dots

Quantum dots typically contain 2 to 5 elements in their shell/core structure, for example, cadmium selenide core is surrounded by a thin ZnSO_4 shell. The toxicity of quantum dots is highly affected by several factors, e.g., size, coating, metal constituting, and surface charge. The toxicity of quantum dots in organs of test animals is also observed in several vivo and vitro studies [81]. Huang et al. reported carbon quantum dots/ ZnFe_2O_4 photocatalyst composites through hydrothermal processes. Synthesized composites possessed a greater transient photocurrent, which results in higher transient photocurrent response. These carbon quantum dots transport energy and act as electron reservoirs during photocatalysis. Toxicity studies revealed low cytotoxicity and good biocompatibility of these photocatalysts with promising roles in air purification [82]. Kammerer et al. reported linewidth measurements on single InGaAs quantum dot transitions. They demonstrated the quenching phenomenon of acoustic-phonon dephasing of

quantum dots, which spectrally separated well from band tail of their respective wetting layers. They achieved a narrow linewidth of few meV through modifying electrostatic environment by decreasing the excess energy used in the excitation processes [83].

iv. Iron Oxide Nanostructures

In natural environment, iron oxide phases carry different information related to chemical and physical conditions of storage and formation of environments. Grained hematite NPs present in the Mars' surface soils show the existence of water by their coexistence and formation of hematite.

Iron oxide and oxyhydroxide nanostructures are important for the ecosystem. Ferrihydrites and oxyhydroxide NPs have large surface to volume ratios, which is important particularly in terms of surface adsorption (Table 7.1). Adsorption capacity is linked with crystallinity and morphology [84].

- **Ferrihydrite:** Ferrihydrite is an iron oxyhydroxide-based mineral with an appropriate composition of $\text{Fe}_5\text{HO}_8 \cdot 4\text{H}_2\text{O}$. It is a poor crystalline

TABLE 7.1

Summary of Naturally Occurring Iron Oxide Nanostructures

Serial No.	Solid Phase	Formula	Shape	Size	Environments	Reference
1	Magnetite	Fe_3O_4	Rhombo-dodecahedron, octahedron	nm to μm	Sediments, anoxic soils and biota	[90]
2	Goethite	$\alpha\text{-FeOOH}$	Acicular	nm to μm	Soils, water (both acidic and alkaline)	[84,90]
3	Maghemite	$\gamma\text{-Fe}_2\text{O}_3$	Different depending on precursor	Not reported	Soils	[90]
4	Hematite	$\alpha\text{-Fe}_2\text{O}_3$	Platy, rhombohedral and rounded	nm to μm	Water and hot dry soils	[90,91]
5	Ferrihydrite	$\text{Fe}_5\text{HO}_8 \cdot 4\text{H}_2\text{O}$	Spherical or irregular	2–10 nm	Near aerated biota, soils and water	[84,87,90]
6	Schwertmannite	$\text{Fe}_8\text{O}_8(\text{OH})_6(\text{SO})_n \cdot \text{H}_2\text{O}$	Pin-cushion or hedgehog like	Not reported	Mine drainage rich sulphate acid	[90,92,93]
7	Lepidocrocite	$\gamma\text{-FeOOH}$	Tabular or lath-like	Not reported	Cool water and wet soils	[90]
8	Akaganeite	$\beta\text{-FeOOH}$	Lath-like, rod or spindle	Not reported	Chloride- or fluoride-rich solutions	[90,91,94]

material with phase containing defects (stacking faults and vacancies), and adsorbed water molecules [85]. Ferrihydrite is wide spread in geological environments. It is found near aerated surfaces of lakes, rivers, soils, and both cold and hydrothermal springs. Naturally present ferrihydrites are linked with different bacterial activities, either iron-reducing bacteria present in anaerobic environments or iron-oxidizing bacteria present in aerated environments. Biotic ferrihydrites can be categorized into abiogenic (byproducts of biological activities) and biogenic (biologically regulated and controlled) origins [86,87].

- **Goethite:** Due to higher thermodynamic stability of goethite, it is commonly present iron oxyhydroxide in soils under ambient conditions. Goethite is also present in different aquatic environments with alkaline and acidic pHs [88]. In certain environments enriched with phosphate, silicate, and organic matters, nanogoethite is reportedly in dominant precipitated phase but usually transformed into other metastable oxyhydroxide (lepidocrocite and ferrihydrite) NPs in water and oils. Since these transformations occur through dissolution–reprecipitation processes inherit precursor phases. After goethite NPs formation, dynamical dissolution–reprecipitation protocols were found to change the morphologies, and this could be catalyzed through Fe(II) [84].
- **Hematite:** Bulk hematite is considered as thermodynamically stable under room conditions, but at nanolevel, higher surface free energies or surface enthalpies make hematite combined with water more thermodynamically stable as compared to nanogoethite. This relative thermodynamic stability reveals that hematite is not the first formed solid phase by nucleation and precipitation but results due to transformation from other oxyhydroxide phases [84,89].

7.4.2 DOSE-RESPONSE ASSESSMENT

In the toxicology studies, the dose is the amount of substance that the body received or is administered. In general, the dose refers to the “dose by mass” (i.e. μg , mg , g). Moreover, a study based on dose-response assessment demonstrated that the biological activity of NPs may not be based on their quality but rather on their functional groups, chemical, and physical characteristics [95]. Quantitative dose-response models have been central attention to the risk assessment of cancer for many years, and dose-response models are used for estimation of risks associated with humans and animals after chemical exposures, characteristically low exposure levels in toxicological studies. These dose-response models entail extrapolation of experimental dose/range below a certain range to estimate the probability of response [96].

7.4.3 EXPOSURE ASSESSMENT

Exposure assessment (EA) plays a vital/key role in the risk assessment of NMs with preconditions of ecotoxicological and toxicological effects and also

explains the routes, uncertainties, and sources for assessment. EA seeks to determine concentrations of pollutants and their bio-usable forms in the environment, focusing on the effects of fate and exposure periods on the target organism. This is useful because measurements reveal the chemical concentrations and physical characteristics of the compound. For NMs, surface charge and size are critical parameters. NMs with the same chemical compositions behave differently when they are released into the environment. Moreover, EA is mainly needed for the assessment of several factors' effects on NPs, such as environmental (pH, viscosity, salinity, temperature), physical (shape, size, area), chemical (reactivity, composition, charge), and biological (molecules, excretion) parameters. EA can be further categorized into three subgroups: environment exposure assessment, occupational exposure assessment, and consumer exposure assessment [95].

7.4.4 ENVIRONMENTAL EXPOSURE ASSESSMENT

The environment has been exposed to NPs through all levels of their life cycle such as storage, transport, material production, water disposal, consumer use, and industrial exercise. The density of released NPs in the environment is determined through mobility in various settings, e.g., water, soil, and air, or via its chemical transformation. Three main factors, i.e. airborne particle's time period, interaction in the environment with different molecules and particles, and distance traveled in the air, play an important role for determination of NPs' fate in the air. The foremost processes for the NPs dynamics in the environment are agglomeration, diffusion, gravitational, and deposition settling. The gravitational settling and the diffusion rate are directly and inversely proportional to particle diameter, respectively [97]. The particle residence time at the nanoscale in the air is smaller ($d < 100$ nm) as compared to medium-sized particles ($100 \text{ nm} < d < 2,000$ nm). It is observed that the deposited NPs cannot be easily re-aerosolized and re-suspended in the environment. Many NPs show high absorption rate and act as catalysts in the environment. The density of NPs in water is determined through different factors, such as water solubility, reactivity with the chemical environment, and its relationship with a different biological process. NPs settle more slowly in the bottom as compared to large particles due to its lesser mass. Moreover, NPs absorb readily to sediment particles and the soil because of its high surface to mass ratio; therefore, they are more liable for removal by water columns [98]. Exposure to bioaerosols present in the environment can have major public health impacts such as acute toxic effects, cancer, infectious diseases, and allergies. Lung function impairment and respiratory symptoms are mostly studied among all. With adverse effects, some of the protective effects after microbial exposure on atopic and atopy conditions have been recommended. In many industrial activities, exposure of bioaerosols can be plentiful such as composting industry, waste recycling, and production of purified enzymes in biotechnological industries to be used further in food industries and detergents. For many biological agents, dose-response relationships have not been defined, while information of

threshold values is sparse. Exposure limits are given for some contaminants such as bacterial enzymes, wood, and flour dust [99].

7.4.5 RISK CHARACTERIZATION

The final step in the risk assessment process is risk characterization. It is usually defined as an assessment of the severity and the incidence of adverse impacts that may occur in a population or environmental compartments. At this stage, all information are collected for the first three risk assessment steps quantitatively. In the risk assessment approach, risk characterization is the ultimate step, in which information of dose response, hazard identification, and exposure procedures are jointly considered for conclusions and the actual possibilities of the risk are compared with the exposed populations [100]. Quantitative risk characterization consists of two parameters: predicted environmental concentration (PEC) and predicted no effect concentration (PNEC). PNEC is a concentration that is less than the substance can cause any hazardous effects, while PEC is the prediction of the concentration of chemical substances in the environment. PEC/PNEC ratio is known as risk quotient (RQ). If the value of this ratio is less than 1, it is considered that no longer risk measurement or testing is required (ECJRC, 2003). If it is greater than 1, additional testing may be initiated to decrease the PEC/PNEC ratio. Important issues in the final step are the assessment data, gestures, and concern associated with every step [101]. A recent report highlighted the possible pathological and toxicological risks to the environment and human health associated with different nanoproducts, especially nano-based controlled drug delivery systems. Synthesized NPs show intermediate supermolecular state between molecular and bulk form of materials. NPs in the size range of 0.1–100 nm possess large surface to volume ratios. NPs' biocompatibility and surface properties depend on the charge carried and chemical reactivity of these particles. Polycationic macromolecules show strong in vitro interactions with cell membranes [102].

7.4.6 SAFETY AND HEALTH HAZARD CONSIDERATIONS

These nanotechnology impacts can be classified in two aspects: (i) potential of medical applications for treating diseases using nanotechnological innovations and (ii) further health risks posed by the exposure of NMs. The NPs' behavior is related to their surface reactivity, shape, and size, along with surrounding tissues [103]. In addition, NPs exposed to tissues and fluids are adsorbed to their surfaces and encounter macromolecules that may affect the enzymes and protein regulatory mechanisms. Other characteristics of NMs that influence toxicity are surface structure, aggregation, chemical composition, absence or presence of functional groups, and solubility. NPs can enter the body through the lungs, digestive system, and skin. This may help to create a "free radical," which can lead to cell damage [104]. Small-size NPs can stick to the cell membrane and enter the cell. NPs attach to the membrane and store in cells, which can impair cell function even in the case of chemically inert NPs, because inert NPs do not break down and react

with other components [105]. Chemical toxicity of manufactured materials can be induced due to ion generation e.g. Cadmium ions can release from CdSe NPs [106]. Carbon nanotubes can easily penetrate the cell membranes. The danger of exposure to NPs is more than just a hypothesis. Studies have shown that some NPs cause damage to the lungs in mice. In the past few decades, the environmental impact of nanotechnology has become an attractive research field. Recently, the harmful effects of NMs on the environment and human health have been quite speculative [107]. The fullerene-based NMs, due to its antibacterial effects on cytotoxicity, accumulate in the mice liver, induce DNA damage, and produce ROS. In addition, inorganic NMs, e.g., SiO_2 , TiO_2 , and ZnO , induce pneumonia in humans and animals [108].

7.5 CONCLUSION AND FUTURE PERSPECTIVE

Today, use of NPs is rapidly increasing in applications ranging from cosmetics to medicine, as well as in chemical, construction, and pharmaceutical industries. Despite their extraordinary properties, still there are toxicity issues linked with NPs when introduced in the living bodies. Humans can be exposed to NPs through various routes/products such as skin products, food and drinking water. Blood stream and respiratory systems are considered other important pathways from their relevant entry. This chapter summarizes that in spite of the adverse effects associated with inorganic NPs, their accumulation rate in bodies is high. This may lead to chronic toxicity due to stimulation of the immune system, which induces inflammatory changes and thus results in malignant transformation of tissues. Moreover, exposure and interactions of NMs can vary in different individuals. Many in vitro and in vivo studies provide data that reveal the adverse effects associated with them. Different entrance routes of NMs in the living organisms and their adverse effects are discussed in detail. However, a crucial point is to enhance the surface modifications through different chemical approaches to make NPs less toxic and safer to use. This can be achieved by standardizing in vitro processes by well-established toxicity profiles. This might be possible by following the standard operating procedures for NPs testing. Future challenges involve the reliable and flexible classification of these NPS according to their toxicological investigations.

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8 Consequences of Nanomaterials on Human Health and Ecosystem

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8.1 INTRODUCTION

The U.S. National Initiative defines the term “nanotechnology” as follows: “Nanotechnology is the control and understanding of matter within the dimension between 1–100 nm, where unique phenomena enable new applications” (Navya & Daima, 2016; Kaphle, Navya & Daima 2018). It is considered as a next-generation technology which promotes economic development through intensive research and findings into advanced products. Nanotechnology has provided a basis for innovation in different arenas from pharmaceuticals to manufacturing sectors (Gottschalk & Nowack, 2011). It has grown at a fast pace within a short duration between the date of discovery and the point of new inventions and commercialization (Xia et al., 2009). Presently, there are about 1814 marketed consumer goods composed of nanoparticles (NPs) which include sports tools, food items, antibiotics, and electronic and cosmetic products, and the number is increasing every day (Vance et al., 2015). The constant development in this field is because of NP’s unique properties like size, surface area, charge, shape, and surface reactivity; it enables its potential applications for pharmaceutical, cosmetics, and medical utilization. The International Organization of Standardization defines NPs as structures whose one, two, or three dimension is in the size range of 1–100 nm (Dwivedi et al., 2015). Materials in nanometer dimensions are not new to the world. They are common in nature, for example, the DNA, proteins, and nanosized particles which are present in the atmosphere. The silver (Ag) and gold (Au) NPs have been used to color stained glass, plane glass, and ceramics (Dowling, 2004).

The NPs have entered into numerous areas like photovoltaic, catalysis, environmental cleanup, space engineering, cosmetic industry, pharmacy, as therapeutics and diagnostics in medicine (such as in specific anticancer therapies), ‘smart’ fabrics which can adjust the temperature according to atmospheric temperature, self-cleaning window glass, and effective chemical processes for the industry. Several nanomaterials are under investigation to improve their functionality and implement into new ones. In the textile sector, many nanomaterials have improved the textile-like self-cleaning, dirt and water repellent, flammability, UV and abrasion resistance, conductive, antimicrobial and antistatic, solvent resistance, and gas permeability properties. The futuristic applications may include the manufacturing of smart materials like food packaging that changes its color after the product’s expiry and space elevator theory by NASA. The advancements in nanotechnology have led to the emergence of nanomedicine which may provide solutions in the diagnosis and personalized medicine for management of complex

diseases (Sanhai et al., 2008). The combined effect of the NPs and advanced techniques led to the development in treating traumatic injuries, preventing diseases, relieving pains, and improvement in human health (Kumari et al., 2020). Along with beneficial effects on the healthcare system, nanotechnology is also found helpful in resolving societal difficulties like environmental pollution (Savage & Diallo, 2005) and energy shortage (Li et al., 2012).

The nanotechnology has been shown to play a significant role in the development of futuristic sustainable technologies for humans and the environment (Oberdörster et al., 2005). However, special care is required to assure that nanotechnology does not become a challenge for the future, similar to the technologies developed during the 20th century. For example, the sudden ozone depletion have taken place in the upper atmosphere due to the release of chlorofluorocarbons. After the first noted hazardous effect, these chemicals which were previously known as “miracle chemicals” were banned from the “non-necessary products” in the early 1990s in the US (<https://archive.epa.gov/epa/aboutepa/regulatory-history-cfcs-and-other-stratospheric-ozone-depleting-chemicals-1993.html>). The utilization of lead in petrol, polychlorinated biphenyls in electronics, and asbestos as a non-flammable building material have led to environmental tragedy (<https://archive.epa.gov/epa/aboutepa/epa-bans-pcb-manufacture-phases-out-uses.html>). Similarly, in the late 1940s, DDT was widely applied in commercial use in the US and was outlawed in 1972 (<https://archive.epa.gov/epa/aboutepa/ddt-ban-takes-effect.html>). Such examples demonstrate the ‘double-sided’ behavior of the nanotechnology. Along with their advantages, several researchers working in the area of health science are concerned about the possible impacts of nanotechnology on the environment and human health.

The production process also defines nanotechnology in another way, i.e., bottom-up and top-down. The top-down approach is the development and fabrication of nanosized structures by cutting down the size of materials using the present methods like etching and machining approaches. The other processing method is bottom-up, also known as molecular nanotechnology, where nanosized materials are built from smaller units, even down to the modification of the individual atoms (Iqbal et al., 2012). To understand the impact of nanotechnology, it becomes necessary to distinguish between free and fixed NPs. The free particles can have a direct effect on health as they are airborne and quickly enter into the body during inhalation, whereas fixed NPs are immobilized and cannot enter the body through inhalation. Another criterion is the difference between NPs which are designed intentionally (engineered NPs) and/or unintentionally (natural as well as anthropogenic NPs) which generally include waste, byproducts, and naturally occurring materials (Figure 8.1).

The unintentional NPs include natural NPs (NNPs), which are formed within the ecosystem, and anthropogenic NPs, which are unintentionally entered into the environment by human activities. In water and soil, NNPs generally result from chemical and biological processes. As of original size selection, NNPs are monodispersed, less toxic, rich in hydroxyl and water groups owing to the

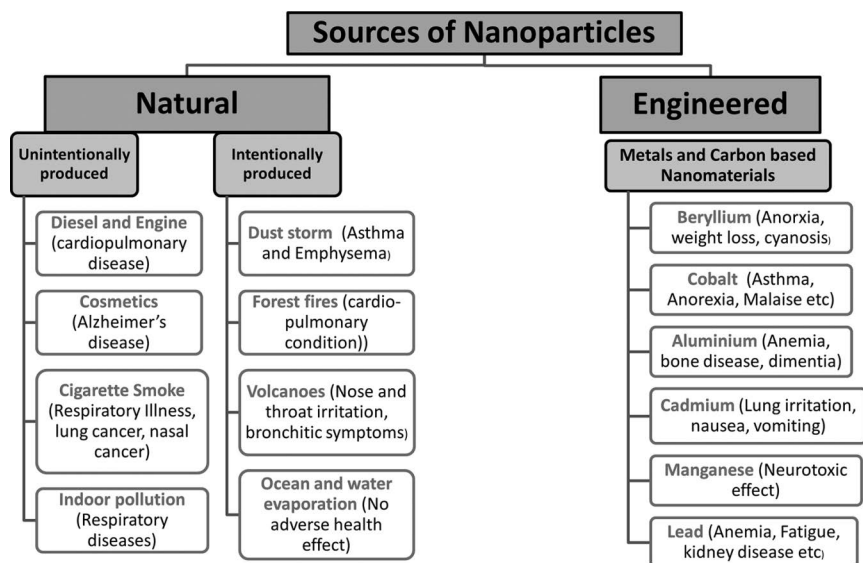


FIGURE 8.1 Different sources of natural and engineered nanoparticles. Natural nanoparticles are categorized into unintentionally and intentionally produced nanoparticles.

higher quantity of hydrous environment, accumulated together into complicated shapes and without any specific ligands on their surfaces (Waychunas, 2009). The prominent categorization of NNPs and all associated methods, which are employed to manufacture these NNPs, is a very difficult and immersive task; it covers each corner of the earth and its chemical entities; and a huge number of diverse mechanisms, processes, and conditions are required for its formation.

Engineered nanoparticles (ENPs) are the man-made or anthropogenic nanomaterials widely used in medical, electronics, energy, and environmental applications. It covers a wide range of materials which includes elemental metals (Ag, Au, Fe, Zn, etc.), inorganic substances like zinc sulfide and oxides, titanium dioxide (TiO_2), graphene, carbon fullerene, and its composites (Mody et al., 2010). Polymeric NPs are composed of the structures of colloidal particles with the size range between 1 and 1000 nm. Polymeric NPs are generally prepared from synthetic as well as natural polymers like poly-D,L-lactide-co-glycolide (Sah & Sah, 2015), polylactic acid (Mahapatro & Singh, 2011), chitosan (Rampino et al., 2013), and gelatin (Elzoghby, 2013; Kumari et al., 2020). Other engineered NPs include silica NPs, micelles, and fullerenes (Kumari et al., 2020). The ENPs are majorly used for improving the drug properties as well as in controlling various complex diseases like cancer. These nanomaterials are also applied as catalysts (Zhou et al., 2018), and the non-reactive metals are studied for their applications in drug development like in thermal therapy for the treatment of tumors (Sharma et al., 2018). The inorganic NPs are already in the consumer market in the form of sunscreens, face mask, and other cosmetic products (Smijls & Pavel, 2011).

The zinc oxide and TiO_2 were used as effective UV filters, in the size range of 20–150 nm BMI, which protects the skin from UV radiation hence acts as desiccants and free radical scavengers (Morganti, 2010; Contado, 2015). The antibacterial properties of nano-Ag are utilized by several cosmetic manufacturers. For examples, nano-Au and -Ag particles are added in toothpastes and claimed to be highly effective in bacterial disinfection in the mouth (Raj et al., 2012). In combination with other NPs like keratin, chitosan, fibrin, and dextran, hydrogels can be used for skin transformation, especially in case of burns (Anjum et al., 2016). The treatment of hyperthermia-induced apoptosis and metastatic bone tumors might be possible by employing iron nanopowders (Matsumine et al., 2007).

Likewise, there are several engineered nanomaterials which are used for the remediation purposes of the environment. For efficient remediation, nanomaterials can be functionalized or surface grafted to target pollutants. The metal and metal oxide NPs are considered as effective absorbents with several advantages like fast kinetics and high adsorption capacity (Santhosh et al., 2016). They are commonly used for remediation because of their highly flexible nature towards their applications in aqueous systems (Das et al., 2015). For example, nano-zerovalent iron is injected into groundwater polluted with chlorinated solvents at remediation sites (Mueller et al., 2012). The nanostructures like TiO_2 , ZrO_2 , CeO_2 , and MnO NPs have been shown to have a high absorption capacity of heavy metals and pollutants in wastewater treatment (Byun & Yavuz, 2016). The nano-adsorbents used for water treatment could be carbon-based (Chowdhury & Balasubramanian, 2014), polymeric, magnetic (Arshadi et al., 2015), and metal-based NPs (Das et al., 2012a). Several nanocatalysts are also widely used for degradation of organic pollutants and water treatment. Several materials like Fe_2O_3 , TiO_2 , ZnO , nano-nickel, zinc, and ferrite are widely used for degradation of organic contaminants in water treatment (Fujishima et al., 2000; Hashimoto et al., 2005; Kurian & Nair, 2015). Besides, nanomembranes are also widely used in wastewater treatment and have replaced the reverse osmosis process due to their low operational cost (Mohammad et al., 2015). Based on their structures, nanocomposite membranes can be divided into two types: mixed-matrix membranes and thin-film membranes. The nano-zeolites, nano-Ag, nano- TiO_2 , and carbon nanotubes are mainly used for these purposes (Qu et al., 2013).

8.2 TOXIC EFFECTS OF NANOPARTICLES ON HEALTH

With the rapid advancement in the nanotechnology and growth of NPs-based products, there is an imperative need to determine their harmful effects on humans as well as the environment. Presently, several engineered NPs are used in several applications which may cause severe issues in future. There are reports available regarding the combinatorial approaches of plant-based molecules with nanotechnology (Kumari et al., 2019). Several nanomedicines are undergoing clinical trials, and therefore approved by U.S. Food and Drug Administration (Kumari et al., 2020). The application of NPs for drug delivery and diagnosis may be a

challenging task. The manufactured sensors consist of highly toxic metals like Te, Cd, and Se. Application of such metals may increase the possibility of their entry into the environment directly or indirectly (Matsuda et al., 2013). Exposure of nanomaterials to staff, customers, and the environment seems unavoidable with the increasing production and the growing number of commercially available nanotechnology-based products (Wijnhoven et al., 2009).

Despite the clear benefits of these small materials, there are unanswered questions like how do the NPs influence the environment and human health. Such key concerns must be resolved prior to the massive manufacture of nanomaterials. There are extensive discussions about how the new properties of nanomaterials might be leading to adverse biological effects (Ray et al., 2009). Therefore, to tackle issues associated with possible health and environment consequences, this chapter addresses recent developments in toxicity issues and environmental impacts of nanomaterials. Nanotechnology's safety impacts are related to the effects of nanotechnological products and devices on health. The impact of nanotechnologies on health can be classified into (i) the potential of nanotechnological advances in therapeutic applications and (ii) the possible health hazards presented by nanomaterial contact. The nanosize of nanomaterials also supports their rapid diffusion into the human body than their large-sized counterparts. Once administered, how these NPs act within the body is among the problems that needs to be addressed. The gradual accumulation of non-degradable particles in body organs is a primary concern. The interaction of NPs with surrounding tissues depends on their size, scale, shape and surface reactivity. NPs can escape from phagocytes which absorb and kill foreign matters in the body. They do so by inducing stress reactions, which cause inflammation and deteriorate the human body's defense against other pathogenic agents. Besides, when exposed to body tissues and fluids, NPs may absorb some of the macromolecules they encounter on their surface, which might further affect the regulatory mechanisms of enzymes and other associated proteins. Additional characteristics of nanomaterials influencing their toxicity are their surface structure, chemical composition, surface load, solubility, aggregation, and presence/absence of functional groups (Nel et al., 2006).

8.2.1 NATURAL NANOPARTICLES AND THEIR TOXICITY TO HEALTH

Toxicity is of a growing concern due to the rapid development and growth of nanotechnology (Nel et al., 2006; Henschke, 2009). There are various types of NPs around the universe which are sorted, mixed, and converted into several other forms. To date, interactions of NPs with the human body are not well known. However, an epidemiological analysis has shown that excess morbidity from fine particles, particularly sulfates, resulted from air pollution in many U.S. cities (Dockery et al., 1993). When NPs are absorbed or ingested by humans, their behavior differs from that of larger particles. They pose a high risk to health because they are likely to be more reactive and toxic than larger particles. NPs may be absorbed into the body via the skin, lungs, and gastrointestinal tract.

When inhaled, it deposits all over the respiratory tract. The particles of ≤ 20 nm show the highest efficiency in deposition (Oberdörster et al., 2005). When ingested, NPs travel into the circulatory and lymphatic systems, as opposed to larger particles. Their toxicity depends on their surface areas and compositions, and they work as catalysts for chemical reactions to surface atoms and molecules. The smallest particles have the most significant specific area of the surface, making NPs extremely reactive. They can cause adverse effects, including oxidative stress, cardiovascular problems such as heart attacks and cardiac rhythm disturbance, and pulmonary inflammation. The health impacts of NNPs are shown in Figure 8.2.

The naturally occurring atmospheric NPs pose a significant threat to human as well as other organisms. The major sources of such particles include natural processing like dust storms, volcanic eruption, and forest fires. These atmospheric particles have diverse impacts on human health. Dust storms are the chief source of NPs on land and desert regions. Studies assisted by satellite images have shown that nano- and micro-sized dust storms and anthropogenic contaminants can spread to thousands of kilometers areas away from their sources. Almost 50% of the aerosol particles in the atmosphere are in size range of 100–200 nm, which originate from dust storms of deserts (Shi et al., 2005). The terrestrial and airborne dust particles are responsible for health problems like asthma and emphysema (Jeevanandam et al., 2018). Dust-borne NPs consist of metals, which

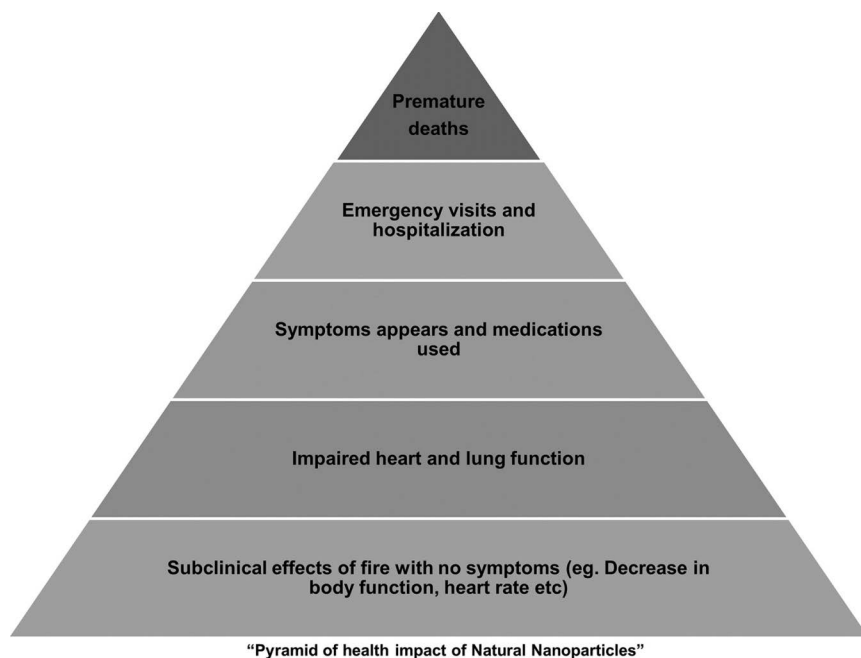


FIGURE 8.2 The representation of pyramid illustrates the impacts of natural nanoparticles and human health.

induces the production of reactive oxygen species and damages lung tissues. The dust storms also lead to the formation of Fe NPs in the clouds, which triggers pH fluctuations and also affects the chemical, atmospheric, mineralogical, and physical properties of the desert in the Saharan region (Shi et al., 2009). These are well-known to irritate Apollo astronaut's eyes and lungs. Some reports showed that dust materials cause pneumoconiosis and fibrosis in rats through the intratracheal route. Astronauts on longer satellite launches are prone to higher risk of respiratory diseases because they are exposed to cosmic dusts. Besides, particulate dusts cause destruction and mechanical failure in airlocks and suits (Buzea et al., 2007).

Moreover, emission of dusts from volcanoes leads to the emergence of fine particles and a huge quantity of aerosols with the size ranging from few micrometers to several nanometers (Ammann and Burtscher, 1990). A sole volcanic eruption releases ash up to 30×10^6 tons of NPs (Taylor, 2002). Particulate debris from volcanic eruptions affect activities of humans, animals, and plants by hindering and scattering the sunlight. The particles released from volcanoes might cause toxicity to humans. The short-term effects of volcanic eruption particles include irritations of the skin, throat, nose, and eyes and bronchial symptoms, whereas the prolonged effects include diseases like Kaposi's sarcoma and podocinids. Podoconiosis occurs due to the absorption of micro- or nanoparticles from the soil via the skin of the feet, resulting in localized fluid accumulation in the lower extremities (Buzea et al., 2007). Kaposi's sarcoma is related to cancers and infection with human herpes virus, which distresses lymph node and blood vessels. This occurs with the induction of NPs into the body (Blundell et al., 1989).

Forest fire activities, which occur either intentionally or unintentionally, lead to the eruption of ash and smoke particles which spreads over a larger area. It has been shown that Asian brown clouds carry and deposit black carbon in huge quantities over the Himalayan glaciers. Ash and smoke particles are known to instigate breathing problems in animals and humans. In patients, smoke consisting of tiny particles may deteriorate pre-existing cardiopulmonary situations. Smoke inhalation was reported to cause 75% of fire-related deaths (Buzea et al., 2007). Sea salt aerosols are the natural NPs produced from seas and oceans either by the evaporation of water or by ejection of water droplets from waves into the atmosphere (Buseck and Pósfai, 1999). The development of CaCO_3 NPs occurred in Lake Michigan due to changes in temperature and weather conditions. These sea salt aerosols act in the transmission of pollutants and microorganisms, which could lead to plant, animal, and human losses and cause adverse health effects.

Additionally, several unintentional NNPs are generated by diverse human activities. These particles have significantly influenced human health. Vehicle exhaust is the key source of atmospheric micro- and nanoparticles in cosmopolitan cities and towns. Among the automobile exhaust forms, diesel engines release particles of 20–130 nm size, while gasoline engines release particles of 20–60 nm

size (Westerdahl et al., 2005). The carbon nanotubes (CNTs) and fibers are found to be released as byproducts during the combustion processes of gas and diesel (Jeevanandam et al., 2018). Delicate particulate matters, especially CNTs of anthropogenic origin, were found in the samples of asthmatic Parisian children's broncho-alveolar lavage fluids. The results showed that CNTs in cells could cause inflammation, granulomatous reactions, and oxidative stress, which results in lung complication such as neoplasia and fibroplasia. These results also demonstrate that humans are frequently exposed to CNTs. The polynuclear aromatic hydrocarbon, benzo[a]pyrene, and carcinogens found in diesel exhaust proved to be highly toxic compared to the gas engine exhaust (Vermeylen et al., 2005). Most of the reported cancer cases in children are because of the exhaust processing, hemolytic reactions, pro-inflammatory, prothrombotic, and myocardial infarction (Riediker et al., 2004). These are few of the health problems detected in humans upon exposure in densely populated urban areas.

Cigarette smoking and building demolitions are among the anthropogenic activities leading to the spread of NPs in the atmosphere. The smoke generated from cigarette consists of a composite mixture of almost 100,000 chemical compounds with a size range of 10–700 nm (Ning et al., 2006). Nano- and micro-particulates $\leq 10 \mu\text{m}$ are dispersed in the atmosphere soon after the demolition of larger buildings (Jeevanandam et al., 2018). Cigarette smoke may lead to prolonged respiratory disorders, genetic changes, cardiovascular complications, pancreatic cancer, asthma exacerbation, and middle ear disease (Rushton, 2004). It is worth noting that the danger of myocardial infarction linked with inhaled NPs can be reversed after discontinuing smoking (Godtfredsen et al., 2003). Respiratory symptoms such as bronchial hyperactivity and cough are found common among firefighters who participated in World Trade Center rescue mission on September 11, 2001 (Fireman et al., 2004).

A poisonous gas that leaked from a polymer factory at Visakhapatnam on May 7, 2020 in Andhra Pradesh, India is one of the examples of release. It immediately killed 11 people and in the early hours affected more than 1,000 people (Siva, 2020). It was similar to the 1984 Bhopal gas disaster, considered as one of the worst industrial catastrophes in the world. Another gas leak incidence in a paper mill of Raigarh (Chhattisgarh, India) industrial town sent seven workers to hospital; besides, three were injured by a boiler blast at a thermal power station in Tamil Nadu, India (John, 2020).

8.2.2 ENGINEERED NANOPARTICLES AND THEIR TOXICITY TO HEALTH

The development and continuous application of ENPs caused a high risk of their release into the environment, which may directly or indirectly affect human health (Figure 8.3). Ag NPs, Au NPs, graphene oxide NPs, ZnO NPs, TiO₂ NPs, and single- or multi-walled carbon nanotubes are examples of several commonly used ENPs. The engineered nanomaterials are quickly becoming a part of our everyday lives as drug delivery systems, cosmetics, biosensors, food packaging,

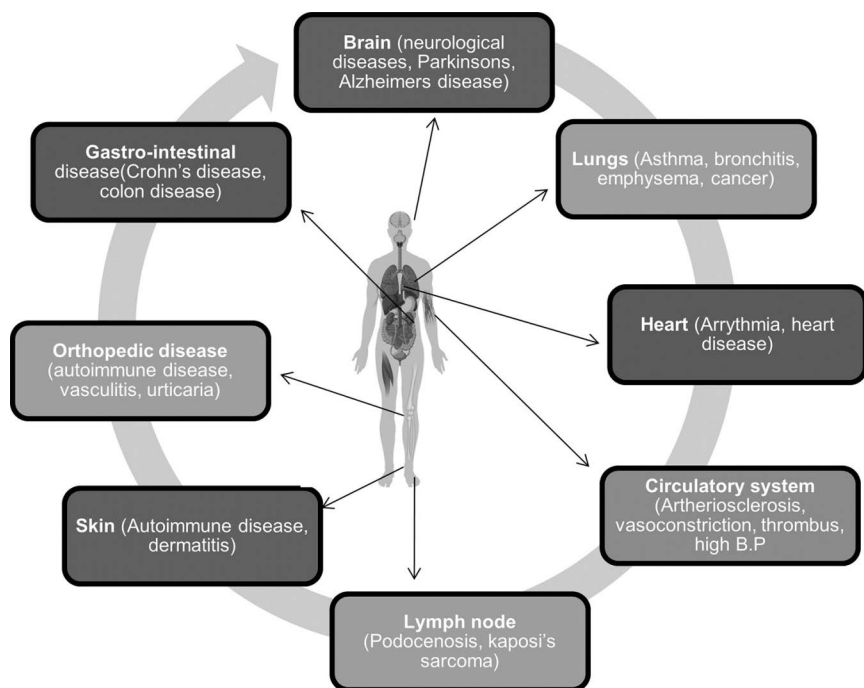


FIGURE 8.3 The diverse effects of natural and engineered nanomaterials on human body parts.

and therapeutics, etc. Simultaneously, their unusual properties eventually contributed to questions about health and ecological toxicity (Maurer-Jones et al., 2013). The metallic NPs are among the most commonly used forms of engineered nanomaterials.

8.2.2.1 Cytotoxic and Genotoxic Effects

The cytotoxicity assessment of nanomaterials is useful in understanding their biological activity and toxicity. The dispersion of NPs is reliant on the medium/solvent used for suspending the particles, which then ultimately affect their cytotoxicity. The cytotoxic effect of several NPs like TiO_2 , SiO_2 , ZnO , and MgO has been explored in Caco-2 cell lines. Most of the NPs, except MgO , showed cytotoxicity (Gerloff et al., 2009). However, SiO_2 NPs were found to be non-cytotoxic in Balb/3T3 mouse fibroblasts (Ubaldi et al., 2012). The cytotoxicity of ZnO NPs on human immune cells was reported, and most likely it was due to the solubility of ZnO NPs into zinc ions after entering into the cells (Shen et al., 2013). The particles showed dose-dependent cellular toxicity in Caco2 and HepG2 cells. In both cell types, an increase in mitochondrial injury and the loss of double-stranded DNA were also observed. A variety of NPs are known to produce cytotoxic properties in *in vitro* and *in vivo* studies which include Ag,

copper, Au, CNTs, and fullerenes. It is suggested that NPs induce cytotoxicity by inducing oxidative stress. Other than oxidative stress, specific mechanisms such as disruption of cell membrane, DNA damage, mitochondrial and lysosomal dysfunction, disruption of actin cytoskeleton because of the released metal ions are also responsible for the cytotoxicity induced by NPs. NPs affect the integrity of cellular membranes *via* direct mechanical disruption of various components (Lin et al., 2010), or by ROS generation and subsequent oxidation of membrane lipids (Voinov et al., 2010). Also, NPs interact with surface receptors of membranes (Sydlik et al., 2006).

Meyer et al. (2010) showed the effects of three different sized Ag NPs with citrate or polyvinyl-pyrrolidone coating on the growth of *Caenorhabditis elegans* (Meyer et al., 2010). The suppression of DNA synthesis and oxidative damage in *C. elegans* have also been observed upon the exposure of Ag NPs (Hunt et al., 2013). Mice studies showed that mostly nanomaterials are accumulated in the liver followed by nasal, inhalation, and intravenous exposures. When nano-Ag (15–20 nm) was orally given to rats for 28 days, it was found that nano-Ag gets accumulated in the liver and spleen at the highest level. The concentration of Ag in the organs was closely correlated with the quantity of Ag ion in the suspension of Ag NPs. It indicates that the intestines in rats were also exposed to Ag NPs (van der Zande et al., 2012). The Au NPs are considered safe, but cellular toxicological impacts still need to be explored (Shukla et al., 2005). The intracellular absorption of colloidal Au NPs of different sizes and shapes was analyzed. The study showed that concentrations of kinetics and saturation are strongly reliant on the physical magnitudes of NPs (Chithrani et al., 2006).

Various methods are used for the assessment of genotoxicity, including oxidative DNA damage, chromosome aberrations, mutations and micronuclei formation, DNA strand breaks, and DNA adducts (Landsiedel et al., 2009). The *in vitro* evaluation demonstrates genotoxicity in Caco2 (human colon) and HepG2 (human liver) cells. The increment in the number of binucleated cells was observed with nano-Ag-induced micronuclei compared to control (Sahu et al., 2016a). A similar study was performed for comparing the genotoxicity of two different sized (20 and 50 nm) nano-Ag. The genotoxicity in both the cells through induced micronuclei was observed when 20 nm-sized NPs were assessed. The larger sized (50 nm) NPs generate a weak response. This study concludes that the size of NPs and types of cells are considered to be critical factors to determine the genotoxicity of nano-Ag (Sahu et al., 2016b).

8.2.2.2 Hepatotoxic and Nephrotoxic Effects

The liver is mainly involved in the synthesis of xenobiotics and detoxification. The liver purifies the blood carrying toxicants and then delivers the purified blood to different parts of the body. The high flow rate of blood to the liver contributes to the enormous toxicant concentration to be administered. The increased exposure and high metabolizing activities make the liver a vital target organ for toxicants. Whenever nanomaterials are inhaled, swallowed, injected intravenously, or absorbed on the skin, it is transported to the liver.

Consequently, nanomaterials may be a hepato-toxicants, and hence, hepatotoxicity testing becomes important (Hillyer & Albrecht, 2001). The toxicity of NPs containing Ag and TiO₂ was determined using BRL 3A as an *in vitro* model (rat liver cell line). The results showed that Ag is extremely cytotoxic in comparison to TiO₂ (Hussain et al., 2005). The toxicity of TiO₂ in mouse liver was due to differentially expressed proteins. Jeon et al. (2011) found 15 proteins through liquid chromatography and mass spectrometry that showed more than a double expression improvement in response to TiO₂ NPs. Among these, 12 proteins were down-regulated and 3 proteins were up-regulated after the treatment of TiO₂ NPs. The 15 differentially expressed proteins can be used in the treatment of acute hepatic damages to detect inflammation, apoptosis, and antioxidant reactions by TiO₂ NPs (Jeon et al., 2011).

The human kidney has been believed as a target organ for toxicity and significant organ for the elimination of nanomaterials. Nephrotoxicity was evaluated in rats when exposed to zinc oxide NPs. It was observed that zinc oxide NPs-induced damages to the mitochondria and cell membranes in rat kidneys led to nephrotoxicity (Yan et al., 2012). Liao and Liu (2012) studied the mechanism of nephrotoxicity in rats when exposed to copper NPs. The necrosis of the proximal renal tubule in nano-copper-induced reins analyzed by profiles of renal gene expression (Liao & Liu, 2012). Such studies suggest that nephrotoxicity may be induced by some nanomaterials.

8.2.2.3 Respiratory and Cardiotoxicity Effects

The airborne nanomaterials are easily exposed to humans *via* inhalation. The NPs are primarily accumulated in the respiratory tract through diffusion (Oberdörster et al., 2004). Inhalation of NPs leads to deposition of NPs in the alveolar section of the rat lungs (Oberdörster et al., 2002). The accumulated NPs cross the biological membranes and enter tissues, which were not exposed to any particles. When TiO₂NPs is inhaled, it is transferred to interstitial lung, resulting in an inflammatory pulmonary overload (Dobrovolskaia et al., 2009). A steady relationship between reactive oxygen species levels, mitochondrial damage, and early apoptosis was observed using Ag NPs when evaluated on A549 (human lung cancer cell line). Pre-treatment with the antioxidant N-acetyl-cysteine significantly decreased the cytotoxicity of Ag NPs (Foldbjerg et al., 2011).

The inhalation of NPs in mammalian models has been shown to cause cardiorespiratory diseases. The continuous inflammation was observed in pulmonary and cardiovascular regions of rats during the intratracheal instillation of CNTs (Chen et al., 2015). The TiO₂ NPs induced apoptosis *via* the pathways mediated by mitochondria, which may be due to an enhanced transition in mitochondrial permeability. This was further followed by the emergence of different factors responsible for apoptosis such as cytochrome c, caspase-3 and caspase-9 activation. The NPs-induced DNA damage was evaluated using the Comet assay, which is further observed from the increment in the length of the DNA tail (Fadda, 2013). When rats were exposed to zinc oxide NPs for a longer

duration, the inflammation in the lungs and myocardial illness were observed (Chuang et al., 2014). DNA injury, inflammation, and apoptosis were observed in the heart of rats after oral administration of zinc oxide NPs. The increase in the levels of CPK-MB, troponin T, and myoglobin indicates the illness of the heart (Baky et al., 2013).

An increase in the serum pro-inflammatory biomarkers, which includes *interleukin-6* (IL-6), tumor necrosis factor- α (TNF- α), and C-reactive protein (CRP), was observed in rats when exposed to zinc oxide NPs. The production of the biomarkers played a fundamental task in cardiac toxicity occurred due to zinc oxide NPs. It has been shown that TNF- α increased the ROS production in the cardiac myocytes culture, resulting in damage to DNA (Suematsu et al., 2003). The TNF- α up-regulation can contribute to an increase of other cytokines, i.e., IL-6, which is a crucial CRP stimulant (de Ferranti & Rifai, 2007). These particles can cause toxicity to the heart by disturbing the homeostasis of calcium. Zinc oxide NPs may affect the permeability of calcium and may lead to the accumulation of Ca^{2+} in the cytosol. This event is consistent with myocardial injury pathogenesis (Hasenfuss, 1998).

Exposure of Ag NPs increases the development of superoxide anions and causes damaging effects in cardiac tissues and also inflammation and oxidative stress in rats. The slowdown of the heart rate was reported in medaka embryos (*Oryzias latipes*) (Ebabe Elle et al., 2013). The accumulation of NPs and deformities was observed in different organs like heart and cardiomyocytes when Ag NPs were dermally applied to guinea pigs (Korani et al., 2013). The liver and myocardial damages were recognized when carbon NPs were exposed to pulmonary regions. This led to a mild modification in serum levels of aspartate aminotransferase, alanine transaminase, creatine kinase, and lactate dehydrogenase. When SWCNTs were introduced into the hypertensive rat's lungs, pulmonary and cardiovascular regions, alterations take place between 24 and 72 hours after exposure of SWCNTs. In treated animals, substantial increases in plasma levels of endothelin-1 and angiotensin I-converting enzyme were observed (Ge et al., 2012). When histamine is released, it can be used as a myocardial ischemia marker and mainly leads to coronary vasoconstriction. Chen et al. (2008) had observed elevated histamine levels. When rats were exposed to silica NPs, it led to acute myocardial injury (Chen et al., 2008).

8.2.2.4 Dermal Toxic Effects

Skin is the largest organ of the human body, and it is most exposed to foreign particles. The solubility, transparency, and color of several cosmetic products were improved by nanomaterials. Nanomaterials majorly used in cosmetics industries are Au, Ag, nanoemulsions, nanocrystals, nanocapsules, dendrimers, fullerenes, liposomes, hydrogels, and solid-lipid NPs (Raj et al., 2012). In several studies, Ag NPs were used to prevent skin cancer and also act as an antimicrobial agent. The potential of nanomaterials during skin penetration is under investigation. Several techniques were used for identifying skin penetration. For example,

in murine model, the *in vivo* skin penetration of quantum dot NPs was estimated using ultraviolet irradiation. They demonstrated low NPs penetration into the skin. It was found that the type of skin, *i.e.*, flexed or unflexed, did not seem to change the entry pattern of nanomaterials, whereas tape-stripped skin interprets quantum dots on the epidermis. It concludes that quantum dots can enter into the dermal layer through skin abrasion (Mortensen et al., 2008).

The two different sized Ag NPs, *i.e.*, 15 and 45 nm, with other cosmetic constituents like aluminum chloride, di-n-butyl phthalate, or methylparaben were analyzed in *in vitro* model pigs after percutaneous absorption. After 24 hours of exposure, the presence of Ag in receptor fluid, which was determined using inductively coupled plasma mass spectrometry, was found to have no statistically significant variations. They noted that 15 nm-sized Ag NPs with methylparaben showed the highest skin penetration ability in the skin of a pig (Domeradzka-Gajda et al., 2017).

8.2.2.5 Some Studies of NPs on Human Health

The epidemiological studies suggest that the nanosized particles of particulate formed during air pollution have a significant impact on cardiorespiratory disease exacerbation and led to a high rate of diseased conditions. The experimental analysis performed in rodents displayed elevated NPs concentration and an advanced reactive surface area per unit mass. In addition, different functionality and chemistry are considered critical for acute and chronic inflammation when NPs are exposed. The accumulation of NPs in organs was found in animal models (Tetley, 2007). Epidemiological studies of air pollution, particularly in occupational environments, highlighted the significance of protecting workers against NPs, which includes exposure quantification and confounding characterization (Peters et al., 2011; Liao et al., 2014).

As ENPs are widely used in diverse areas, they are modified for a particular purpose. So, these nanomaterials need significant attention. Therefore, epidemiological studies and experiments mainly concentrate on the impacts of ENPs on human health. The ENPs was utilized for a short duration with restricted groups of people exposed to it, and the length of exposure was reasonably limited. The workers were mainly exposed to various ENPs (silica dioxide, CNT, nano-Ag, nano-resin, and TiO₂) at the same time. The results ranged from changes in oxidative stress markers (Liao et al., 2014; Pelclova et al., 2017). Elevation in the levels of several inflammatory biological markers, cardiovascular markers, and local and systemic pulmonary damage markers was noticed (Liao et al., 2014; Wu et al., 2014; Lee et al., 2015; Fatkhutdinova et al., 2016). The symptoms of allergic dermatitis, sneezing, and changes in functional parameters in the lung occurred (Liao et al., 2014). Based on the available information on ENPs exposure level of workers, it is assumed that ENPs exposure is correlated with human health. However, it suggests that multiple types of biomarkers may be affected by staff being exposed to ENPs, and this information will open diverse areas for further study. Therefore, the present knowledge makes a platform for future epidemiological studies.

8.3 TOXIC EFFECTS OF VARIOUS NANOPARTICLES ON THE ENVIRONMENT

The continuous utilization and release of NPs into the environment presently leads to their accumulation in the atmosphere. Once these NPs are released into the atmosphere, this leads to a massive exposure in different ways. In addition to health toxicity, environmental issues are also concerned. There are a number of particulate matters dispersed in the air. Once they are condensed and agglomerated and attain a certain size, they will fall by gravity. The water bodies may also serve as a reservoir and transportation of NPs. Thus, ecotoxicity studies of NPs are limited and challenging task (Gautam et al., 2016). Presently, there are no such standardized protocols for determining the ecotoxicity of NPs at diverse environmental categories like soil, water, and air (Baysal et al., 2017).

The increased application of NPs will lead to emission of different varieties of release sources into the environment (Nowack & Bucheli, 2007). NPs can enter into the atmosphere by different pathways such as (i) emission from raw materials and nano-based products formation, (ii) emission from utilization of NPs, and (iii) emission from disposed nano-based products (Gottschalk et al., 2013; Foss Hansen et al., 2016). The emission of NPs takes place either directly or indirectly *via* different sources like wastewater treatment plants or landfills, and effluent release from the wastewater treatment plants. Until now, the material flow models that follow NP life cycle are employed for determining the emission and environmental concentration levels. The different calculation models presume that NPs will be emitted either to waste streams or directly to separate compartments of the environment. The emission of NPs can be controlled by (i) weathering or ageing (Kaegi et al., 2010; Bressot et al., 2017), (ii) fate of NPs during use (Kaegi et al., 2011; Kinsinger et al., 2015), and (iii) waste management system (Keller et al., 2013). The global estimation indicates that soils (8%–28%) and landfills (63%–91%) receive the huge share of NP emissions followed by release into the aquatic ecosystem and air (7% and 1.5% of the production volume). Such type of analysis allows determining applications with high environment implication.

8.3.1 NATURAL NANOMATERIALS AND THEIR TOXICITY TO THE ENVIRONMENT

Every living organism is surrounded by NPs, directly or indirectly. The release of NNPs into the environment and their toxic effects are shown in Figure 8.4. They are available in nature in huge quantity as they are formed in several natural processes including photochemical reactions, forest fires, volcanic emission, and erosion either by plants or animals. The anthropogenic sources of NPs are industries, cars, combustion, etc. Besides, several natural processes like dust storms, landslides, volcanic emission, forest fires, and heavy rainfalls lead to the formation of a vast amount of nano-particulate matters that affect the air quality index worldwide. Few examples are ferrihydrite NPs moderated by microbial activity, or the development of halide and hydrous sulfate NPs from the sea spray evaporation, and nanosized mineral fragments developed by forming carbon NPs from

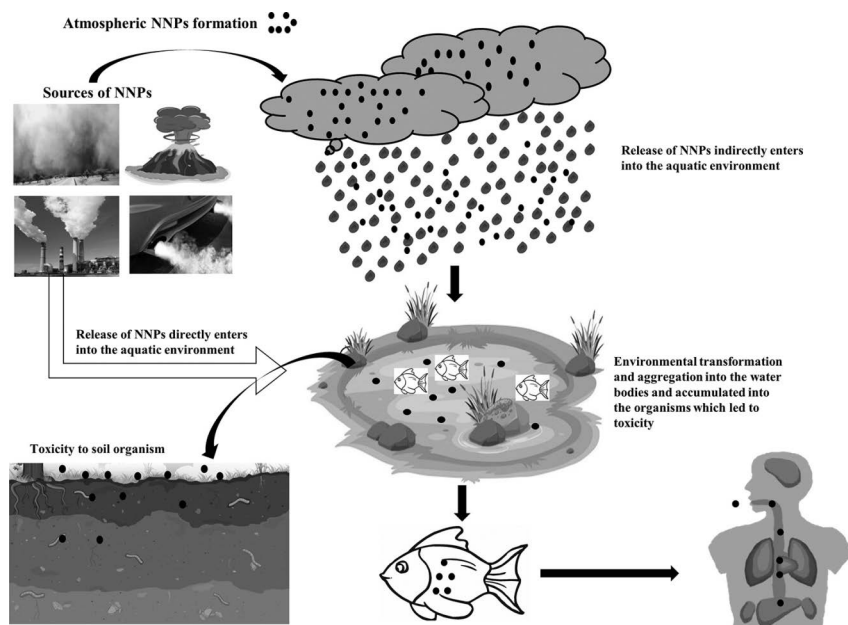


FIGURE 8.4 The release of natural nanoparticles into the environment and its toxicological effects on environment which directly or indirectly affect the human health.

the biomass combustion or wind erosion on deserts (Hochella et al., 2015). The nanostructures almost cover the atmosphere (elemental composition present in the whole troposphere), hydrosphere (lakes, rivers, groundwater, oceans), lithosphere (soils, rock, lava, and magma), and biosphere (microorganisms like bacteria, fungi, and viruses) (Hochella et al., 2015).

There are several NPs that are formed naturally into the environment. Fullerenes are one of the examples that are produced in cooking and combustion, and also an area of research. The unintentional NPs are generated due to combustion activities, like the formation of soot from power generation, diesel exhaust, and natural weathering of iron oxides and silicates (Masciangioli & Zhang, 2003). The processes that are involved in the formation of the natural NPs intentionally and unintentionally are shown in Figure 8.5. In rural areas, NPs are generated from the oxidation of volatile compounds obtained from biogenic or anthropogenic sources. In contrast, in urban regions, the sources are car exhausts, industries, and catalytic converters (Schneider et al., 2005).

8.3.1.1 Impact of Atmospheric Nanoparticles

Among natural NPs, atmospheric NPs are major NNPs released in the environment either intentionally or unintentionally. About 10% of aerosols are generated by human activities, and the remaining 90% are released from a natural source (Taylor, 2002). The most important constituents of the worldwide atmospheric

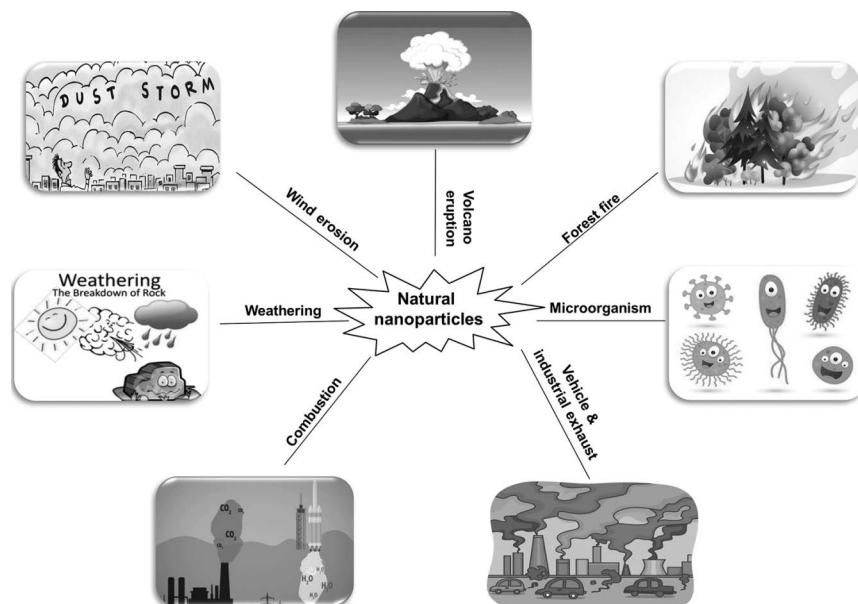


FIGURE 8.5 The natural and anthropogenic activities involved in the formation of natural nanoparticles intentionally and unintentionally.

aerosols exist in declining mass abundance: mineral aerosols erupted from the wind erosion with a negligible constituent, i.e., <1%, 16.8 Tg (1 teragram = 10^{12} g) from volcanoes, 3.6 Tg from sea salt, 3.3 Tg from natural and anthropogenic sulfates, 1.8 Tg from products of biomass burning, 1.4 Tg from soot, 1.3 Tg from natural and anthropogenic non-methane hydrocarbons, 0.6 Tg from natural and anthropogenic hydrocarbons, and 0.5 Tg from biological debris (Buseck & Pósfai, 1999).

There are about 90% of aerosol NPs present in the environment which are generated naturally. The NPs obtained from the dust storms, soil erosions, forest fires, and volcanic eruptions surround humanity since pre-historic times. Among atmospheric NPs, dust storms are one of the single leading source of NPs release into the environment. The extensive passage of mineral dust and man-made pollutants from large continents has been the new area of extreme investigation. There are approximately 50% of the troposphere atmospheric aerosol particles are minerals produced from deserts (Shi et al., 2005). The size of dust storm particles differs from 100 nm to few microns with about one-third to a half of the dust mass being lower than 2.5 microns. The particles ranging from 100 to 200 nm can attain 1500 particles/cm³ concentrations (d'Almeida & Schütz, 1983). Dust particles coated with polluted particles act as condensation nucleus for the development of warm clouds and efficient ice nucleus for the formation of a cold cloud. However, this ability is mainly dependent on the composition, shape, and size,

which ultimately depend on the parent soils, emissions, and transport mechanism. Alterations in the microphysical composition of clouds transform their capacity to absorb radiation from the sun and scatter it back to space, thus indirectly affecting the energy reaching on the surface of the earth (<https://www.ipcc.ch/report/ar5/wg1/clouds-and-aerosols/>). These facts are also evident from the satellite images and lead to the production of airborne particles and particulate matters in the size range from micro- to nanometers.

The frequent dust storms were observed on the Indo Gangetic plains during the pre-monsoon season (Kumar et al., 2015). Drastic changes were observed in the suspended particulate matters, air quality, and weather pattern after a dust storm in the Indo Gangetic plains (Sarkar et al., 2019) (Figure 8.6). There are several stories that showed the impact of dust storms on the environment. The dust has many adverse effects on agriculture, including reduced crop yields by covering seedlings, loss of plant tissues, decrease of photosynthetic activity, and increase of soil erosion. The unintentional dust deposits include stuffing the irrigation canals, covering the transportation tracks, and altering the water quality of rivers and streams. Also, dust has a significant effect on land and air transportation due to reduction of visibility. Dust also affects the output of solar power plants, particularly on those that depend on direct solar radiation. Deposition of dust on solar panels is the foremost concern for operators of solar power plants. Making

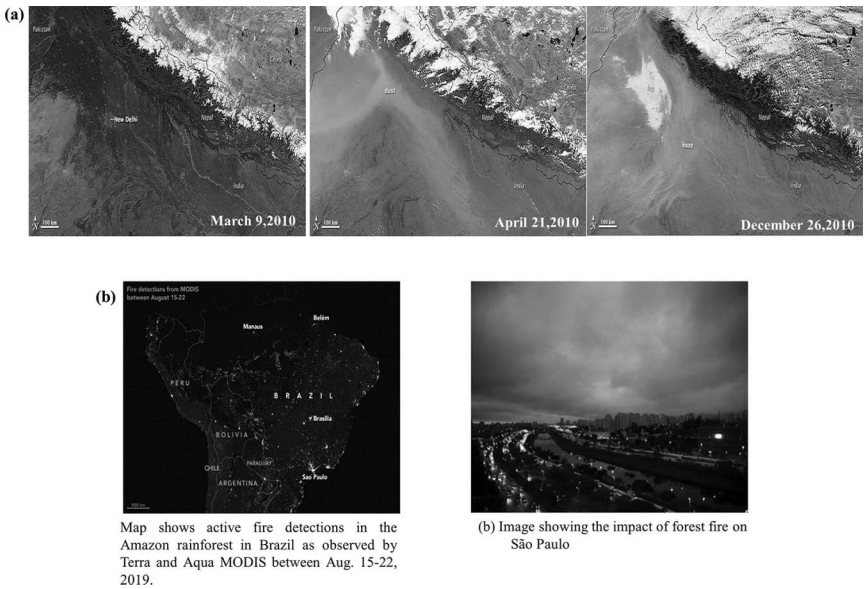


FIGURE 8.6 (a) The impact of dust storms on Indo gangetic plains throughout the year, (b) The impact of the amazon forest fire on the environment. (Note: The images are taken from the moderate resolution imaging spectroradiometer (MODIS) on NASA’s Terra and Aqua satellite.)

the solar collectors dust free and preventing blockage of incoming radiation consume labor and time.

Forest fire is becoming more prevalent and affecting the forest ecosystem and natural resources. It is mainly generated by lightning and human activities. Forest fire composed of smoke and ash, which can have the ability to spread up to thousands of square miles. The smoke generated from forest fires composed of several pollutants like particulate matter (PM), carbon monoxide (CO), methane (CH₄), volatile organic compounds, nitrogen oxides (NO_x), and polycyclic aromatic hydrocarbons (PAHs) (Naeher et al., 2007). These components may degrade air quality, which can be a global issue (Johnston Fay et al., 2012). One big example of forest fires that significantly affected the environment is Amazon rainforest fire on August 28, 2019. As the fire continues to sweep through the Amazon rainforest in Brazil, the flames were captured by the International Space Station and confirmed to be the most active fire in Brazil since 2010 (Figure 8.6) (Rabbie, 2019). According to Brazil's National Institute for Space Research, there are about 26,000 fires recorded in the Amazon until August 2019. The Amazon rainforest is considered to be the "lungs of the planet." It serves as a carbon "sink" absorbing the carbon dioxide and provides 20% of the world's oxygen.

Among several natural sources, volcanoes have caused mass extinctions, degradation of large ecosystems, global climate change, and severe effects on human civilization. When volcanic eruptions take place, a mixture of gases and particles ranging from nano-scale to microns enter into the environment. Sometimes, the amount of diverse ashes and particles released into the atmosphere reaches very high levels to affect the whole earth for several years. The preliminary effect of particles on the upper atmosphere is that they reflect the incoming solar radiation and absorb outgoing radiation, which leads to cooling of the earth. A single eruption can emit 30 million tons of ashes and particles into the atmosphere and can spread over the sky that can turn day into complete darkness and reduces the visibility. One of the largest outbreaks of volcanic eruptions in the recorded history is 1815 eruption of Mount Tambora, Indonesia, with an estimated death of 10,000 inhabitants. This eruption emitted about 150 km³ of debris into the air, which dropped the global temperature by 3°C, causing drastic weather conditions around the world for three years. Several challenges were faced in these years due to this volcanic eruption such as crop damage, famines, and diseases (Evans, 2002). NNPs in the form of ashes are dangerous in many ways; for example, they can affect entire communities and regions. The minute abrasive particles can enter inside the airplane engines and affect the blades of the turbine, causing engine failure. For example, the 2010 eruption of Eyjafjallajökull, Iceland, led to the production of an ash cloud, which became a big reason for the cancelation of about 100,000 flights (Alexander, 2013). When mixed with the rainfall, volcanic ash particles turn into heavy, cement-like sediments that can damage the roofs of buildings. In 1991, the eruption of Mount Pinatubo, Philippines collapsed the roofs of houses, schools, and hospitals in many provinces (Spence, 1996).

Several livestock and mammals have been killed by the eruption flow along with famine, forest fires, and earthquakes. It affects both aquatic and terrestrial lives. Increase of acidity, turbidity, temperature of water and change in the quality of foods in water bodies can cause complete damage to the aquatic ecosystem. Similarly, the eruptions influence the bird's migration, roosting, flying ability, and feeding activities. Mount St. Helens is one of the examples that disturbed many lives and ecosystems. According to the Washington Department of Game, about 15 mountain lions, 200 black bears, 300 bobcats, 1,400 coyotes, 5,200 elk, 6,000 deer, and 11,000 hares died from the pyroclastic flows during the 1980 eruption (Mitchell, 1986). These are few natural sources that contribute to a greater extent for the production of diverse forms of NNPs. Depending on environmental conditions and strength of sources, the number and concentration of NPs in the atmosphere differ up to five or more orders of magnitude (from 10^2 to 10^7 particles/cm) (Kumar et al., 2010). Few effects of natural nanoparticles on environment and health are mentioned in Table 8.1.

8.3.1.2 Impact of Naturally Produced NNPs

There are several NNPs produced by mechanical, chemical, biological, and thermal processes individually or in combinations in the environment. A recent study suggests that about a thousand teragrams of NNPs were produced per year only from the biogeochemical practices, which is comparatively lower to the mass of ENPs production (Hochella et al., 2015). Inorganic sulfides (H_2S and HS^-) are the main components of the biogeochemical cycle, which includes mining water, hydrothermal vents, sewage treatment plants, sediments, and terrestrial soil. The high temperature in the hydrothermal vents leads to the emission of metal and sulfur into the ocean, and they react with each other, and metal-bearing sulfide NPs are formed and stay suspended (Yücel et al., 2011). When the Ag NPs undergo oxidation, Ag^0 transforms into Ag^+ , which undergoes sulfidation to generate insoluble Ag_2S , which ultimately transforms to core shell Ag_2S NPs (Kaegi et al., 2013). This phenomenon has been observed in wetland sediments and sewage sludge (Lowry et al., 2012). The AgCl NPs can be converted to Ag_2S NPs in the sewer system (Levard et al., 2012). Not only metal sulfide NPs, but different ZnS , Ag_2S , CdS , and CuS NPs have also been originated in the sulfidic environment (Schaffie & Hosseini, 2014).

Different types of metal NPs are considered to be an emerging class of NNPs which are formed by the natural processes and have significant ecological impacts (Adegboyega et al., 2014). Examples of NNPs include natural Ag NPs in coastal water areas and Ag mine activity (Akaighe et al., 2011). The metal salts have the ability to react with an array of vitamins, sugars, antioxidants, and plant extracts and even with natural organic matters (NOMs) and reactive oxygen species (ROS), which leads to the production of metal particles in the environment (Yin et al., 2012; Hou et al., 2013; Yin et al., 2014). NOM is a mixture of polysaccharides, proteins, humic materials (HM), and few necessary elements of surface waters, rivers, sediments, soil, and groundwater at different concentrations (Aiken et al., 2011). The groups involved with the HM are aromatic carbon,

TABLE 8.1
Impact of Natural Nanoparticles (NNPs) on Environment and Health

Impact of NNPs on Environment		References
Natural Nanoparticles (NNPs)	Impact of NNPs on Health	
Soot obtained from burning of biomass; particulate matters and carbon NPs from unprocessed fuel led to formation of Asian brown clouds	Melting of glaciers Increased atmospheric temperature Disturb the monsoon pattern which affects the agriculture	Gustafsson et al. (2009), Engling and Gefencsér (2010), Griffin (2007)
Atmospheric nanoparticles	Reduction of hydroxyl radicals and ozone layer depletion	Manning et al. (2005), Wilson et al. (2007), Rohrer and Berresheim (2006)
Smoke particles (ash, partly consumed fuel, and liquid droplets)	Serious visibility hazard Temporary changes the air quality Disturb the habitat Negative impact on aesthetics Changes the environmental quality	https://www.auburn.edu/academic/forestry_wildlife/fire/effects.htm
Indoor particles obtained from homes using dirty solid fuels (such as charcoal, waste wood, dung, coal, and crop wastes) on open fireplaces, cooking stoves	Increases the breathing problems and respiratory disorders	Leung (2015)

phenolic group, and conjugated double bonds. The HM is sub-categorized into humic acid (HA), fulvic acid (FA), and humin. HA is a high-molecular-weight constituent, insoluble in low pH. FA is a low-molecular-weight constituent, soluble at a broad range of pH, and humin is non-soluble in all pH conditions. ROS encloses $^1\text{O}_2$, O_2^- , H_2O_2 , and $^{\cdot}\text{OH}$, produced *via* photochemical and Fenton-like reactions in naturally present surface waters (Yin et al., 2012, 2014). It was shown in some studies that interaction between Ag^+ and NOM leads to formation of Ag NPs. The functional groups present in NOM like methoxyl, phenolic-OH, aldehydes, ketones, enolic-OH, quinones, and thiols lead to reduction of Ag^+ ions to Ag^0 with a characteristic band at 400 nm (Akaighe et al., 2011; Adegbayega et al., 2013). Similarly, the production of Au NPs takes place when Au(III) is heated at 65°C in rivers with a characteristic band at 520 nm (Yin et al., 2014). The seaweed, *Sargassum muticum*, leads to the formation of Au NPs in the seawater environment (Bavelloni et al., 2015).

The concentration of naturally generated Ag and Au NPs is too little to allow for direct exploration of their toxicity. Thus, researchers have carried out very few studies related to the toxic effects of NNPs. For example, the toxicity of natural Ag NPs (produced by the reduction of Ag^+ with organic matter) was determined by estimating the minimum inhibitory concentrations (MIC) against Gram-positive and Gram-negative bacteria. Due to the differences in the results of these two bacterial strains, the toxic effect of Ag NPs also differs (Adegbayega et al., 2014). When the MIC values of ENPs (sodium dodecyl sulfate-coated Ag NPs, and polyvinylpyrrolidone-coated Ag NPs) were compared to the NNPs against bacterial strains, the MIC against Gram-negative bacteria was lower as compared to Gram-positive bacteria (Adegbayega et al., 2014). This study suggests that the toxicity of Ag NPs differs with the difference in species.

Along with this, most interestingly, NNPs have high MIC values compared to ENPs, suggesting the less toxicity behavior of NNPs, which may be due to the organic coating in the natural environment. Another possible reason is that NOM is less toxic than the surfactants or polymers used for the formation of ENPs. Thus, it may be concluded that atmospheric NPs significantly affect the environment, compared to NNPs generated via different naturally occurring processes.

8.3.1.3 Impact of Unintentionally Produced NNPs

In addition to the intentionally formed NNPs, there are several unintentionally produced NNPs that affect the environment. The sources of unintentionally created NNPs are industries, car exhaust, mining, etc. The various combustion processes lead to the emission of NPs, particularly in traffic-influenced environment, reaching concentrations higher than 10^5cm^{-1} (Al-Dabbous & Kumar, 2015). They are usually emitted from engines of vehicles. Besides, they are found to be released from the combustion of fuel oil (Happonen et al., 2013), coal-based production of heat and power (Mylläri et al., 2019), and flue gases emitted from industries (Al-Dabbous & Kumar, 2015). The unintentionally released NPs have a

huge impact on climate change and vegetation. For example, the Visakhapatnam gas leakage led to emission of a poisonous gas that has a high impact on the atmosphere and vegetation.

8.3.2 ENGINEERED NANOMATERIALS AND THEIR TOXICITY TO THE ENVIRONMENT

Until 2015, the broad utilization of nanomaterials in the diverse areas like medicine and high technologies has resulted in an increment of 1.5\$ trillion (Nel et al., 2006). As the ENPs' application in science and technology expanded, understanding of their eco-toxicological impacts is highly essential (Ju-Nam & Lead, 2008). ENPs are automatically discharged into the waterways and soil, and ultimately reach the ocean (Ju-Nam & Lead, 2008). The unintentional and uncontrolled ENPs are released into the environment and discharged to waste streams (Weir et al., 2012) from eroding structures (Kaegi et al., 2010), exhausts, and manufacturing sites (Engeman et al., 2013). These ENPs can enter several water bodies and ecosystem, and pose a serious risk of exposure for wildlife and humans. ENPs are generally entered into the surrounding environment during their production, application, and disposal. There are different sources from which a diverse forms of ENPs are released and affect a diverse forms of ecosystems. Some of the examples are as follows.

8.3.2.1 Impact of ENPs on Different Ecosystems

ENPs in the aquatic environment may transfer secondarily with water, accumulate in the biosolids of wastewater treatment plants (WWTPs), and pass to soils of agricultural lands during biosolids application (Nowack et al., 2012). ENPs in terrestrial ecosystems may transport with soil pore water horizontally or vertically (Sagee et al., 2012), and thus expand their distribution from one site to another. The aquatic ecosystem, especially surface water, is easily contaminated by ENPs because they are considered to be their sinks. ENPs are added directly from consumer waste and wastewater, and indirectly from groundwater or soils and atmospheric deposition (Scown et al., 2010). The entry of nano-Ag and nano-TiO₂ occurs from the textile industry during washing, where WWTPs are transported via sewage (Walser et al., 2011; Windler et al., 2012). If not separated with WWT, then ENPs could run through effluent-receiving waters. Continuous release of ENPs has a great impact on the microbial communities of the aquatic ecosystem. These microbes are involved in many biogeochemical processes, and hence, changes in aquatic microbial communities have raised concerns about the effect of ENPs. A decrease in the diversity of bacterial communities was observed when exposed to polymer-coated nano-Ag in seawater (Doiron et al., 2012). Modification in the structure of the bacterioplankton community were observed when exposed to functionalized Ag NPs in a natural water system. It was also observed that phylotypes were xenophobia (disappeared when exposed to ENPs); phylotypes were recovered after

early rapid decline; tolerant phylotypes (not influenced by exposure to ENPs) and those stimulated by exposure to ENPs (Das et al., 2012b). The other ENPs may affect the microbial environment under certain environmental conditions of a natural aquatic ecosystem, but this concept is less studied. For example, when surface water microorganisms are exposed to nano-TiO₂ in combination with natural bacterial aggregation, damaging of the membrane of planktonic and (less impact) biofilm entrapping cells initiated (Battin et al., 2009).

Besides the ENPs uptake or adsorption to microbial diversity, they are transmitted to other higher organisms through predation with interference for biomagnification and bioaccumulation within the aquatic food chain. Accumulation of CdSe quantum dots has been reported in bacteria *Pseudomonas aeruginosa*, which biomagnified within the grazing ciliate protozoa *Tetrahymena thermophila* in a simplified food web (Priester et al., 2009). The trophic transfer of quantum dots from ciliates to rotifers was observed, but without indication for biomagnification (Holbrook et al., 2008).

The effect of ENPs on soil enzymatic activities is different in diverse soils. For example, it was reported that TiO₂, ZnO, and Zn inhibit the various soil enzyme activities which include catalase, peroxidase, β -glucosidase, acid phosphatase, and dehydrogenase (Kim et al., 2011). Another study demonstrates the stimulated soil invertase and urease activities by Fe₃O₄ and γ -Fe₂O₃ (He et al., 2011). The carbon-based NPs, e.g., fullerenes, have no impact on enzymatic activities (Tong et al., 2007), and MWCNTs considerably suppress enzymatic activities, e.g., β -glucosidase, phosphatase, cellobiohydrolase, β -N-acetylglucosaminidase, and xylosidase (Chung et al., 2011).

The personal care products are daily requirements for everyone's life. The ageing process of TiO₂ nanocomposites used in the sunscreens was reported in the literature in detail. It has been found in one of the studies that TiO₂ nanocomposites and sunscreen formulations go through swift modification in water, promoting the dispersion of NPs in the aqueous environment. On the basis of salinity, pH, and NOM concentration in seawater, colloids may last longer and stable, aggregated or suspended, and settled out of the water column. These colloids are generally mobile and interact with planktons and other feeders, while ENPs that settled down may be integrated into deposits and further eaten by the animals living in the bottom layer of water.

The state of Hawaii introduced a new law in 2018 to ban sunscreens that contain octinoxate (ethylhexylmethoxycinnamate), oxybenzone (benzophenone 3), and ultraviolet radiation (UVR) filters. About three-fourths of the sunscreens are composed of these chemicals, which causes bleaching of coral reefs. After that, Key West, Florida, and US Virgin Islands have also banned the sale of such sunscreens and found effective starting January 2021 (Florida) and March 2020 (US Virgin Islands). This had led to a rise in concerns related to the environmental impacts of UVR filters, which include oxybenzone, 4-methylbenzylidene camphor, octinoxate, and octocrylene.

Several problems were faced during the organic filter removal in WWTPs, which is due to the low aqueous solubility and high lipophilicity of UVR filters (da Silva et al., 2015; Ekpeghere et al., 2016). An *in vitro* study showed that oxybenzone causes coral reef bleaching, induces ossification, and deforms DNA in the larval stages. In a similar study, oxybenzone was identified in about 0.8–1.4 µg/l concentration in seawater. The study also demonstrated the median lethal concentration of oxybenzone for seven different coral species ranging from 8 to 340 µg/l over 4 hours of exposure (Downs et al., 2016).

The carbon nanoparticles (CNPs) have been used in diverse areas, due to their enchanting physiochemical properties and large surface area to volume ratio. CNPs are gaining increased attention from researchers. CNPs include carbon nanotubes (CNTs), graphene, graphene oxides, and fullerenes. These are discharged into the environment from diverse media in different amounts, which have a negative effect on the distinct biotic stratum of the environment (Mottier et al., 2017). Similar to others, they can pass into the environment at any level of formation, controlling, use, and discarding. CNPs are considered to be the least biodegradable NPs that are non-soluble in water and lipophilic in nature, which leads to their accumulation in the aquatic food chain (Wu et al., 2006). The first nanotoxicity study on fish was conducted using CNTs (fullerenes) on different species of fish, namely, fathead minnow, Japanese medaka, zebrafish, rainbow trout, and largemouth bass. The dose range between 0 and 1 ppm of C₆₀ and SWCNTs resulted in lipid peroxidation localization in the brain, enhanced gill irritation, aggressive behavior, and modification in the brain pathology (Smith et al., 2007). Similarly, a drastic effect on trophic-level microorganisms was observed with increase in growth inhibition rate, when the benthic diatom *Nitzschia palea* was exposed to traces of MWCNTs and DWCNTs particles (Verneuil et al., 2015). Thus, the aquatic ecosystem is greatly affected by CNPs.

Not only the aquatic environment, but the terrestrial ecosystem is also greatly affected by NPs. Plants are generally important in eco-nanotoxicology due to their direct exposure to CNPs present in the terrestrial ecosystems and atmosphere. CNPs adhere to the leaves and aerial fraction of plants, while roots absorb waterborne and soil-associated CNPs. It was reported that the plants having high leaf area indexes are profoundly affected by airborne CNPs, which increases their chance to enter into the trophic webs (Köstner, 2001). The interaction between the *Catharanthus roseus* cells and fluorescent MWCNTs was carried out to detect the phytonanotoxicity to characterize the cell uptake and localization (Serag et al., 2011). The higher bioaccumulation of CNTs and DNA damage were observed in earthworms when the nanotoxicological studies were conducted using ¹⁴C-labeled CNTs (Petersen et al., 2016). Effects of engineered nanoparticles on the environment and health are mentioned in Table 8.2.

TABLE 8.2
Impact of Engineered Nanoparticles (ENPs) on Environment and Health

Engineered Nanoparticles (ENPs)	Impact of ENPs on Environment	Impact of ENPs on Health	References
Carbon nanotubes	Enter the food chain and disturb the whole environment	Apoptosis, diminished cell viability, lung toxicity, oxidative stress, cell growth retardation, inflammation of the skin, etc.	Helland et al. (2007), Albini et al. (2015)
Nanosilver	Toxicity to mammals and non-mammals, soil nematodes and aquatic life	Particles when inhaled can lead to inflammation in the cardiovascular and respiratory systems Some known health effects include asthma complications, chronic bronchitis, and respiratory tract irritation and infections	Yu et al. (2012), Quadros and Marr (2010)
Fullerenes	Impact on soil species and enzymes; toxic effects on fish, invertebrates, algae, and soil microbial community	Enhance ROS production, genotoxic	Shinohara (2012)
Polymeric nanoparticles	A possible hazardous environmental risk factor	Oxidative stress, inflammation, changes in the morphology and functioning of cells	Viswanath and Kim (2016)
Silicon based nanoparticles	Potential hazardous environmental exposure factor, harmful ecosystem impact, etc.	Cardiovascular effects, cytotoxicity, and oxidative stress improvement	Murugadoss et al. (2017)
TiO ₂ nanoparticles	Disturb the aquatic ecosystem, soil microbial community	Increased oxidative stress, genotoxicity, neurotoxicity, respiratory toxicity	Shah et al. (2017), Simonin et al. (2016)

(Continued)

TABLE 8.2 (Continued)
Impact of Engineered Nanoparticles (ENPs) on Environment and Health

Engineered Nanoparticles (ENPs)	Impact of ENPs on Environment	Impact of ENPs on Health	References
Gold nanoparticles	Au NPs cause biotoxicity and necrosis as they have the potential to be internalized in the exposed plants	They are able to enter and leave cells, destroy blood vessel intruders, and even search for errors in DNA. They can be deposited in the respiratory system and have toxicity affected by nanostructure due to high surface area, high surface activity, irregular morphology, small diameters, or degradation after deposition into smaller particles	Ray et al. (2009)
Quantum dots	DNA damage due to oxidative stress induced by CdTe QDs was revealed as the main reason for the prophase activation	Skin penetration, cytotoxicity, and genotoxicity	Ahmad et al. (2012)
MnO ₂ , TiO ₂ and carbon nanoparticles	These NPs may aggregate on long time storage and may impart toxicity as the bare surface is more reactive	Possibility to enter the brain	Viswanath and Kim (2017)
Copper oxide nanoparticles	Inhibit seed germination, reduce photosynthesis and respiration rate, decrease shoot and root lengths of the crop plants, cause oxidative stress, and impart serious deleterious effects in the tissues and affect fish growth and development	May cause skin damage	Rajput et al. (2018), Gupta et al. (2016), Zanoni et al. (2019)

8.4 FUTURE PROSPECTIVE

Understanding of the potential effects of NPs on the environment and health has increased significantly in the recent past. Initial experimental and analytical studies are urgent requirements to analyze the health and environmental impact of NPs. Many relevant knowledge gaps still exist, which leads to several toxicity issues. In future, more parameters need to be established, so that a proper release of NPs into the environment can be decided. In many studies, the properties of NPs were not adequately characterized or reported. Thus, based on well-characterized nanomaterials, it would be feasible to disclose the significant endpoints and distinguish the measurement in the categorization of NPs. Standard methods must be developed to explore the toxicity of different NPs. The studies examining the release of NPs from industries are minimal. To overcome this issue, research is needed to enhance the representativeness of the release process. In the case of NNPs, various techniques such as transformation, metal binding and uptake of contaminants related to NNPs are used. Therefore, the data generated under lab conditions do not mimic the natural environment, which makes the study challenging. Toxicity studies using cell lines are available, but due to broad concentration of NPs, different cell lines, and culture conditions, the mechanism was not fully understood. Analytical strategies that permit *in situ* monitoring, optimize formation procedures in real time, minimize cost and waste, and provide mechanistic information are required. The framework of green nanoscience should be developed to design, produce, and use of greener NPs across a diverse range of sizes, compositions, functionalities, and shapes.

To assess the safety of nanomaterials, scientists and researchers need to build approved models that can predict the emission, passage, modification, deposition, and uptake of NPs in the environment and toxicity assessment in humans. To predict the impact of nanomaterials, these models should relate to the physicochemical properties of nanomaterials. The models should determine the biological impact and degradation of nanomaterials. These models are essential in designing NPs that can determine the toxicity to human health and the environment. Many reports state the application of surfactants for controlling the shape and size of nanomaterials. Therefore, an alternative needs to be developed as it is considered to be toxic. Subsequently, more research is needed for building of such methods. The development and application of such techniques will provide research opportunities and challenges for this community in future.

8.5 CONCLUSION

The application and scope of nanomaterials in health and the environment are increasing. Thus, nanotoxicology has attracted great attention in the past few decades. The toxicity of NPs varies with the types of materials and with different

experimental studies for similar NPs. It could be due to the deficiency of characterization and investigation of physicochemical characteristics and difference in testing techniques. Knowledge gaps still exist regarding the toxicity of natural and engineered NPs. NPs are unpredictable and unique that the present toxicity assessment protocols cannot fulfil the toxicity criteria.

Presently, there are no guidelines related to the toxicity assessment for NPs. Different regulatory bodies like EMA, ICH, OECD, and FDA are still facing problems in deciding the proper protocols for safe utilization of NPs. The dose selection is among the major issues for the toxicity assessment. Hence, dosage assessment after thorough characterization may fill the gaps and help in defining the generalized guidelines for the toxicity of NPs. Besides, the eco-toxicological threat is still untouched. The study for the proper estimation of the release of nanomaterials still needs attention as it ultimately affects the environment and human health. A proper toxicity check should be established before the release of any waste material into the environment as it may contain numerous toxic NPs which may hinder the food chain. Comprehensive understanding of the toxicity of NPs will provide valuable understanding of the effects and could make it possible to establish ecofriendly NPs and promote sustainable nanotechnology.

ABBREVIATIONS

NNPs:	natural nanoparticles
ENPs:	engineered nanoparticles
NPs:	nanoparticles
Ag NPs:	silver nanoparticles
Au NPs:	gold nanoparticles
TiO₂ NPs:	titanium nanoparticles
NOM:	natural organic matter
ROS:	reactive oxygen species
HM:	humic materials
HA:	humic acid
FA:	fulvic acid
MIC:	minimum inhibitory concentrations
CNPs:	carbon nanoparticles
CNTs:	carbon nanotubes
SWCNTs:	single-walled carbon nanotubes
DWCNTs:	double-walled carbon nanotubes
MWCNTs:	multi-walled carbon nanotubes
IL-6:	interleukin-6
TNF-α:	tumor necrosis factor alpha
CRP:	C-reactive protein

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9 Conceptual Understanding of the Mechanisms of Nanotoxicity and Safety of Nanomedicines

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9.1 INTRODUCTION

The application of nanotechnology has gained popularity especially in the field of medicine, but due to their potential toxicity, their usage has been so far limited. Nanotoxicology, therefore, studies the toxicity of nanoparticles (NPs) to further recognize and evaluate the health risks related to their applications (Lanone and Boczkowski, 2006). NPs possess physicochemical properties that contribute to their enhanced toxicological effects due to their size, surface area, and shape, which give them the advantage to be used as nanomedicines (Donaldson et al., 2004).

Human exposure to nanomaterials could be by oral ingestion, injection, inhalation, and/or the dermal route (Medina et al., 2007). For example, studies have shown that carbon nanotubes (CNTs) caused persistent interstitial inflammation and epithelioid granulomatous lesions in the lungs (Morimoto et al., 2013). Besides, ceramic NPs used for drug delivery exhibited oxidative stress and cytotoxic activity in some organs like lungs, heart, liver, and brain, along with carcinogenic effects (Singh et al., 2014).

The accumulation and potentially adverse effects of NPs in various organs have discouraged their use as drug delivery systems and in molecular diagnostics. Therefore, understanding the mechanistic toxicity of nanomaterials may help to situate applications for biomedical purposes.

NPs have distinct applications due to their characteristics such as very minute size and increased surface area (Khaled et al., 2019). NPs have a great prospect in the development of new and distinct products such as biosensors. It has a wide application in medicine, sporting goods, cosmetics, etc. (Luyts et al., 2018). There is an information gap as to the level of NPs released to the environment in terms of usage and spillage and also the impact of such NPs at different concentrations to the environment and humans. This gap creates the need to study NP toxicity in eco- and biological systems (Khaled et al., 2019). NPs toxicity could exhibit varying effects in certain individuals, and those with lower immunity or lower antioxidant response seem to be more affected by NP toxicity than others, for example, the elderly, children, invalids, or people with heart conditions or diabetes (Luyts et al., 2018). With recent development in research and application of NPs, the toxicity of these particles is a major consideration in several recent studies.

In the field of medicine, there has been rapid advancement in the application of NPs for diagnostic, therapeutic, and theranostic purposes as tools for drug delivery for enhanced effectiveness in treatments of varying conditions. Because of the

continuous growth, application, and promotion of nanomedicine and nanomedical research, there is a need to ascertain the safety of nanomedicines in long-term application (Huang et al., 2017).

9.2 DNA DAMAGE

Recently, there have been increasing studies reporting that a large number of NPs are more likely to cause genotoxicity. Molecular mechanisms underlying nanotoxicity have been shown to include oxidative DNA adducts, breakage of DNA strand, and gene expression alterations profiles, chromosomal fragmentation, and induction of gene mutations (Li et al., 2008). At present, the assessment for genotoxicity mainly adopts the cell line model, rat model, and plant model. Reports revealed that 1 nM concentration of gold nanoparticles (AuNPs) exhibited DNA damage with reduced expression of DNA repair (Singh et al., 2009). Also, exposure to metal oxide NPs confirmed the formation of oxidation-induced DNA adducts and fragmentation (Bhattacharya et al., 2009). In cells, the response mechanism to DNA damage includes initiation of DNA repair or invoked cell death and cell cycle arrest. There is usually activation of effector molecules in response to DNA damage; p53 is an example of an effector molecule that is essential for DNA repairs and cell cycle arrest, thus preventing mutagenic events (Lane, 1992).

In human breast carcinoma cells, the significant increase in p53 level and upregulation of the p53-downstream effectors was observed with exposure to cadmium-telluride quantum dots (QDs) (Jeng and Swanson, 2006). Also observed is altered expression of DNA damage responsive genes due to NP exposure (Li et al., 2008). If the DNA repair mechanisms could not repair the DNA damage, the cells initiate apoptosis. Apoptosis is programmed cell death with a highly regulated pathway involving several signaling molecules. NPs, especially that of metal oxide with particle size ranging from 30 to 45 nm such as ZnO, Fe₃O₄, TiO₂, Al₂O₃, and CrO₃ induce apoptosis (Choi et al., 2008).

The study of genotoxicity after acute oral exposure to cerium oxide nanoparticles (CeO₂ NPs) in female Wistar rats using micronucleus test, chromosomal aberration assay, comet assay, reduced glutathione content, lactated hydrogenase activity, and alkaline phosphatase activity assay was conducted by Kumari et al. (2014). CeO₂ NPs and cerium oxide microparticles (CeO₂ MPs) at 1,000 mg/kg bw (body weight) induced an obvious increase in % tail DNA damage in the liver. However, due to their greater surface area, CeO₂ NPs appeared to be more genotoxic than CeO₂ MPs. Ghosh et al. (2016) used the plants (*Nicotiana tabacum* and *Allium cepa*) model, animal (Swiss albino male mice) model, and human lymphocytes model to show that silver nanoparticles (AgNPs) cause chromosomal aberrations and DNA damage, thus raising concern about the AgNPs.

9.3 CYTOTOXICITY

The summary of cytotoxicity of selected NPs is presented in Table 9.1.

TABLE 9.1
Cytotoxicity of Selected Nanoparticles

NP	Cell Line	NP Concentration	Exposure Duration	Toxicity	Author
AgNPs	HepG2 and A549 cells	12.5–200 mg/ml	24 hours	AgNPs induce oxidative damage via elevation in MDA, 8-oxo-dG, and 8-epi-PGF2a levels and upregulation in stress-inducible HSPA1A and HO-1	Xin et al. (2015)
AuNPs	Bovine endothelial cells, GM7373	0–50 mM	96 hours	Exposure to 50mM induces toxicity detected in XTT-assay	Taylor et al. (2012)
Quantum dots	Human embryonic kidney cells (HEK293 cell line)	10, 7.5, 5, 2.5, and 1.0 μ M	24, 48, and 72 hours	CdTe/CdS QDs are toxic to the HEK293 cells QD cytotoxicity is linked to their surface structures and physicochemical properties	Zheng et al. (2012)
TiO ₂ NPs	RBL-2H3 cell	0.1–1 mg/ml	24 hours	TiO ₂ NPs induce oxidative stress and can contribute to cytosolic Ca ²⁺ signaling as a result of increase in both cytosolic Ca ²⁺ concentration and histamine secretion in a dose-dependent manner	
Carbon nanotubes (CNTs)	Monocyte-derived dendritic cells (MDDC) and monocyte-derived macrophages (MDM)	0.005, 0.01, 0.02, 0.03, and 0.04 mg/ml	24 hours	After SWCNT and MWCNT exposures, there is an increase in tumor necrosis factor (TNF)- α and interleukin (IL)-8 in a dose-dependent manner at concentrations up to 0.02 mg/ml	Clift et al. (2014)
Titanium dioxide nanoparticles (TiO ₂)	HUVEC cells	5, 10, 20, and 40 μ g/cm ²	24 and 72 hours	TiO ₂ NPs inhibit proliferation of cells strongly and induce apoptotic death, oxidative stress, and necrotic death starting at 5 μ g/cm ²	Montiel-Dávalos et al. (2012)
TiO ₂ nanoparticles	RAW264.7 (ATCC # TIB-71) cells	10–100 nm	48 hours	TiO ₂ nanoparticles lead to radicals generation and reactive oxygen species (ROS)-mediated cytotoxicity after photoactivation	

9.3.1 CYTOTOXICITY OF CARBON NANOTUBES

Carbon allotropes are classified as multi-walled carbon nanotubes (MWCNTs) and single-walled carbon nanotubes (SWCNTs). CNTs are fiber shaped with unique thermal, physical, and electrical qualities with a great application in bio-medical science (De Volder et al., 2013).

Investigation revealed ROS generation by CNT leading to the activation of ROS-associated intracellular signaling pathways such as mitogen-activated protein kinase (MAPK), activator protein-1 (AP-1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in mesothelial cells (Clift et al., 2014). Reports suggest that release of cytokines IL-1 β , TNF- α , IL-8, and IL-6 from macrophages and mesothelial cells is stimulated by MWCNTs (Murphy et al., 2011).

9.3.2 CYTOTOXICITY OF TITANIUM DIOXIDE NANOPARTICLES

Titanium dioxide nanoparticles (TiO₂ NPs) have had widespread application as they can confer opacity and whiteness in products such as food additives, pharmaceutical preparations, paints, and cosmetics (Yin et al., 2013). Recently, greater attention has been given to TiO₂ NPs because of their photocatalytic killing effect on cancer cells (Sha et al., 2013). Although there are still controversies as regards the potential cytotoxicity mechanism of these non-soluble TiO₂ NPs, some studies consider TiO₂ as a natural nanomaterial.

Potential toxicities of TiO₂ NPs such as genotoxicity, immunotoxicity, and oxidative stress have also been reported, although the toxicity mechanism is not yet clear (Gerloff et al., 2012; Montiel-Dávalos et al., 2012). The toxicity of TiO₂ NPs could be attributed to the size-dependent interaction between intracellular biomolecules and NPs, and unlike other NPs such as ZnO, TiO₂ NPs do not produce toxic ions (Xiong et al., 2013). These interactions result in cytokine release, mitochondrial depolarization, intracellular calcium influx, plasma membrane leakage, and ROS generation (Chen et al., 2012).

The influence of TiO₂ NPs' size was investigated on phototoxicity in a study by Xiong et al. (2013). The results revealed a significant high cell mortality rate after UV light photoactivation in cells treated with 10 nm TiO₂ NPs compared with larger particles (20 and 100 nm particles). Thus, there might be a converse relationship between particle size and phototoxicity. Furthermore, a higher generation of mitochondrial superoxide was observed in the cells treated with 10 nm photoactivated particles compared to 20 and 100 nm particles. Indeed, the smaller particles have the higher surface area and hence the high cytotoxicity induced.

9.3.3 CYTOTOXICITY OF QUANTUM DOTS

QDs are promising NPs composed of colloidal semiconductor nanomaterials. They possess exceptional optical properties which make them attractive candidates for *in vivo* imaging instead of fluorescent dyes. Their unique properties

include narrow emission, broad absorption, high photostability, and high fluorescent quantum yield (Osiński et al., 2010).

Just as we have it in other NPs, QDs' cytotoxicity is dependent on particle surface coatings, concentration, redox activity, size, mechanical stability, charge, and shape. Some studies have investigated the toxic level of uncoated core CdSe or CdTe QDs. The major mechanisms of toxicity of these inorganic NPs include formation of radicals and Cd^{2+} ions existing in QDs structure. The formation of radicals by QDs produces highly reactive molecules. Therefore, oxidation occurs when UV or visible light is used in QDs photoactivation. QDs can be excited by a photon of light, which produces an excited electron that forms singlet oxygen when the electron reacts with a molecular oxygen. Therefore, free radicals are produced when the singlet oxygen reacts with biomolecules or water. Another mechanism based on Cd^{2+} ions existing in QDs structure occurs as a result of Cd^{2+} generating toxic effect due to interference with DNA repair and substitution for physiologic Zn (Hoshino et al., 2011; Zheng et al., 2012).

9.3.4 CYTOTOXICITY OF GOLD NANOPARTICLES

Gold nanoparticles (GNPs) have increasingly attracted technological and scientific interests as one of the promising NPs due to their chemical stability, ease of synthesis, and excellent optical properties. GNPs have become diagnostic tools for cancer treatment because of the aforementioned properties (Dykman and Khlebtsov, 2012). It is essential to evaluate the cytotoxicity of GNPs due to their applications in biomedical sciences. The cytotoxicity of GNPs is dependent on their surrounding ligands, shape, and size (Taylor et al., 2012). Anisotropic GNPs compared with the isotropic ones are prone to oxidation as a result of their exposed surface areas (Pissuwan et al., 2011).

An investigation using an *in vitro* model on Balb/3T3 mouse fibroblasts showed cytotoxic effects of 5 and/or 15 nm GNPs. Different studies have examined the intracellular distribution and the uptake of NPs to further understand the cytotoxic difference between the two sizes of GNPs. The results indicated no toxicity for the 15 nm GNPs in Balb/3T3 fibroblasts, while the 5 nm GNPs had cytotoxic effects. This observation could be explained by the number of NPs taken up by cells which is higher for the 5 nm GNPs compared to the 15 nm particles (Coradeghini et al., 2013).

9.3.5 CYTOTOXICITY OF SILVER NANOPARTICLES

Silver nanoparticles (AgNPs) have antimicrobial properties, and hence, they can be used as broad-spectrum antimicrobial agents in consumer products such as textiles, food products, electronics, cosmetics, and household appliances (Chaloupka et al., 2010). Recently, AgNPs have found their use as biosensors, drug delivery systems, and imaging contrast agents (Zhou et al., 2011). Oxidation of AgNPs to Ag^+ ions can be the reason for the cytotoxicity of these NPs, which are toxic to cellular components and biological systems (Xin et al., 2015). Investigation of

NPs in aqueous systems reveals that AgNPs are more toxic compared to the bulk Ag due to the release of H_2O_2 from AgNPs and the presence of dissolved oxygen. Furthermore, studies also revealed that AgNPs generate more ROS than bulk Ag (Batchelor-McAuley et al., 2014).

9.4 NANOTOXICITY AND CELLULAR DEATH MECHANISMS

The cellular effect of nanotoxicity is presented in Figure 9.1.

9.4.1 APOPTOSIS

Apoptosis was initially described as a distinct form of cell death under normal physiological conditions (Lockshin and Zakeri, 2001). The signs accompanying this cell death include the formation of apoptotic bodies, inter-nucleosomal DNA fragmentation, cell shrinking, and chromatin condensation (Jayakiran, 2015). Apoptosis is a cellular suicide categorized into either extrinsic or intrinsic processes. In mammalian cells, mitochondria-controlled signaling determines the intrinsic pathway, whereas plasma membrane death receptors determine the extrinsic pathway (Lockshin and Zakeri, 2001). Also, the intrinsic apoptosis pathway may be either caspase-independent or caspase-dependent signaling. However, the extrinsic apoptosis pathway is caspase-dependent signaling. TNF receptor superfamily members mediate extrinsic apoptosis. The caspase proteases propagate cell death, inflammation, and proliferation/differentiation.

The initiation of the cellular signaling cascade culminates in apoptotic cell death, and oligomerization of the cell surface receptor occurs when ligands (TNF-related apoptosis-inducing ligand-TRAIL, TNF-R, and Fas ligand) bind to death receptors (TRAIL receptor 1 or 2, Fas receptor, and TNF-receptor I) (Meier and Vousden, 2007). Fas ligation on targeted cells results in the assembly of the multi-protein complex at the plasma membrane. Fas-associated death

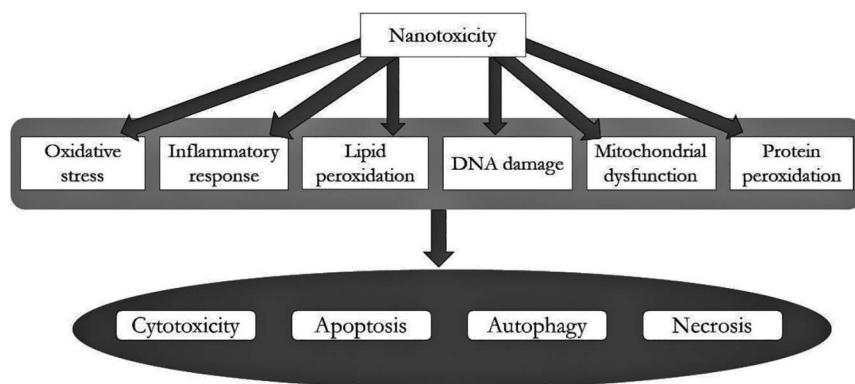


FIGURE 9.1 Cellular effect of nanotoxicity.

domain-containing protein, also called FADD, is the adaptor protein that binds to Fas through its death domain, leading to pro-caspase-8 and -10 recruitment. This produces a complex called death-inducing signaling complex (DISC) and also activates caspase.

The intrinsic apoptotic pathway is initiated by the permeability of the mitochondrial membrane, oxidative stress, and growth factor deprivation (Birnbaum et al., 1994). The superfamily of BCL2 proteins are classified as anti-apoptotic and pro-apoptotic, and can control mitochondrial integrity. The pro-apoptotic family members include BAK1, BAX, BAD, BID, PMAIP1, and BBC3, while BCL2, BCL2A1, BCL2L1, and MCL1 are the anti-apoptotic proteins (Black et al., 2004). CYCS (cytochrome c, somatic) is released into the cytosol following mitochondrial permeabilization. Apoptosome formation is therefore initiated when CYCS binds to apoptotic peptidase activating factor 1 (APAF1). The apoptosome helps to recruit and activate CASP9, which subsequently leads to CASP3, CASP6, and CASP7 activation, and eventually results in apoptosis (Jayakiran, 2015).

9.4.1.1 Apoptosis: Effects of Nanoparticles

Several studies involving the apoptotic effect of CNTs have been reported. Srivastava et al. (2011) reported that apoptosis was induced at different doses in A549 lung carcinoma cells by MWCNTs, as estimated by DNA laddering and nuclear condensation, while no observable cell death in A549 cells was reported by Thurnherr et al. (2011). After six months of chronic exposure to SWCNTs (0.02 µg/mL), human lung epithelial cells displayed a significant increase in cell proliferation. The cells after exposure for a long time showed induced tumor formation and the cells became resistant to apoptosis (Wang et al., 2011).

Tyurina et al. (2011) used an oxidative lipidomics approach in mice exposed to SWCNTs which revealed selective lipid peroxidation and an increase in apoptotic neutrophils in the lungs. These suggest that the *in vivo* exposure to SWCNTs leads to the activation of apoptosis signaling pathways, and therefore, deleterious effects of CNTs can be mitigated by agents that fight against selective lipid peroxidation (Tyurina et al., 2011)

9.4.2 AUTOPHAGY

Autophagy is primarily a cellular mechanism for cytoprotection to cope with nutrient deprivation and ATP deficiency for survival. Autophagy ensures homeostasis by facilitating the removal of damaged or dysfunctional organelles, thereby allowing the recycling of cellular building blocks (Levine and Klionsky, 2004). There are two basic types of autophagy: selective process and nonselective process. The selective process can be further classified based on mechanism or sequestration.

The mammalian target of rapamycin complex 1 (mTORC1) is a key autophagy checkpoint. Thus, cellular energy sensed by AMP-activated kinase (AMPK) and signaling is integrated into the PI3K/Akt pathway by mTORC1. Growth factor receptors such as the insulin receptor inhibit autophagy, then activate PI3K/Akt,

and promote mTORC1 activity through TSC1/TSC2 inhibition. However, autophagy is downregulated by activated mTORC1 through autophagic proteins complex phosphorylation (ULK1/2) and induces nutrient starvation through mTORC1 inhibition (Levine and Klionsky, 2004).

9.4.2.1 Autophagy: The Effects of Nanoparticles

Different researchers have proposed that NPs could act as activators of autophagy. Seleverstov et al. (2006) showed the induction of autophagy in human mesenchymal stem cells by QDs in a size-dependent manner. Stern et al. (2008) also showed that cytotoxic CdSe QDs caused autophagy in porcine kidney cells. Other NPs that activate autophagy *in vitro* or induce cell death include iron oxide NPs, GNPs, and fullerenes (Wu et al., 2011). An inquiry, therefore, is necessary to know if the nanosized particles are taken up by the cellular defense or whether NPs cytotoxicity is caused by an autophagic cell.

Also, with the involvement of mTOR pathway, autophagic cell death was triggered by iron oxide NPs (50 nm) in cancer cells found in the human lungs (Khan et al., 2012). Recent *in vivo* studies have shown the pathological role of autophagy. It was noted that 3-methyladenine (3-MA) which is an autophagy inhibitor ameliorated acute lung injury in mice, and inhibition of Beclin-1 also rescued A549 cells from cell death which generated PAMAM dendrimer-induced cytotoxic effects mediated via autophagic cell death (Li et al., 2010).

Moreover, 3-MA reduced carboxylic acid-SWCNT-induced cell death and also reduced the acute lung edema in mice. COOH-functionalized SWCNTs also induce formation of autophagosomes in A549 cells (Liu et al., 2011). Detection methods for autophagy must take into account the dynamic nature of the process (Galluzzi et al., 2012). Specifically, steady-state detection methods may not give an accurate estimate, because they do not discriminate between decreased off-rate (inhibition of autophagosomal lysosomal fusion) of autophagic activity and increased on-rate (enhanced rates of autophagy). Ma et al. (2011) provided evidence that 1,050 nm size GNPs induced accumulation of autophagosomes through lysosomal impairment. However, the authors suggested that the accumulation of autophagosomes is a result of autophagy flux blockade rather than autophagy induction. The basal autophagy blockade and autophagy induction have contrasting effects on degradation within the cell: autophagy induction increases degradation, while the basal autophagy blockade inhibits it (Ma et al., 2011). Hence, the lysosomal function of the cell could be interfered by certain NPs which could lead to "lysosomal storage disease."

9.4.3 NECROSIS

For a very long time, necrosis was assumed to be just an uncontrolled and accidental cell death or due to non-specific cell injury resulting from trauma, but recent research has shown that necrosis can be regulated. Various pathological conditions that induce necrosis include trauma, neurodegenerative disorders, viral or bacterial infection, ischemia, and exposure to toxins (Degtarev et al., 2005).

The regulated necrosis can also be called “necroptosis.” Necroptosis was initially explained by Yuan and co-workers to explain a particular form of regulated necrosis that is activated by ligation of the death receptor and blocked by an inhibitor of necroptosis called necrostatin-1 (Vandenabeele et al., 2010). They also proved that RIP1 kinase (an association adaptor of death domain receptor) functions as a target of new anti-necroptotic compounds in cells (Degterev et al., 2005). There are different subprograms of regulated necrosis which include RIP1-independent and RIP3-dependent cases of necrosis (Zhang et al., 2009).

Stimulation of death receptors such as TNFR1 induces necrosis and depends on the activities of receptor-interacting protein kinases (RIPK). Necrosome, which can induce either necroptosis or apoptosis, contains caspase-8, FADD, RIPK1 and RIPK3, and also TRADD (TNF receptor-associated death domain-containing protein). Generally, apoptosis is triggered by caspase-8 activation, but the absence or blockage of caspase results in RIPK1 and RIPK3 phosphorylation which leads to regulated necrosis.

The initial stage of the cell death pathogenesis involves a sub-lethal phase that allows the cells to either recover (reversible) or pass into an irreversible stage (permeabilization of mitochondrial membranes) (Galluzzi et al., 2009). Morphological changes observed at reversible stages include degeneration of feathery, vacuolar degeneration, and change of hydropic or cloudy swelling (Tavakol et al., 2017). The irreversible stage involves a separation between the inner and outer membranes of the mitochondria, which is then followed by the irreversible loss of oxidative phosphorylation activities (Galluzzi et al., 2009). In the final stages of necrosis, the cytoplasm takes on a homogeneous eosinophilic appearance and also loses its contents coupled with irregularities in the organelle’s membrane, vacuoles formation, matrix density increase, and mitochondrial swelling. In the nucleus, chromatin patterns are seen with karyolysis (complete chromatin disruption), karyorrhexis (nuclear fragmentation), and pyknosis (chromatic condensation) (Chaabane et al., 2013).

9.4.3.1 Necroptosis: Effects of Nanoparticles

There are different forms of NP-triggered cell death reported in the literature. A report suggested that in macrophages, several forms of cell death such as caspase-1, pro-inflammatory, and pyroptosis were triggered by 10nm carbon black NPs (Reisetter et al., 2011). Also, induced cell death was triggered by 4nm germanium NPs and was subsequently inhibited by necrostatin-1 in another study which shows an example of NP-induced regulated necrosis (Ma et al., 2011). Finding discriminative biochemical markers has remained a challenge in studying necroptosis.

There have been inconsistent reports about necrotic effects of NPs due to focusing on the loss of cell viability without aiming at the exact cell death mode, while in some conditions, apoptosis is followed by secondary necrosis, which leads to incorrect interpretation (Vandenabeele et al., 2010).

For instance, titanium dioxide (TiO₂) NPs affect both size and crystal structure by inducing cell death mechanism in mouse keratinocyte cells. In a study, it was revealed that apoptosis was induced by TiO₂ NPs with rutile crystal structure,

whereas in a size-independent manner, necrosis was elicited by TiO₂ NPs with 100% anatase crystal structure (Pan et al., 2007). In another study, GNPs showed size-dependent cytotoxicity; smaller-sized NPs (<1.4 nm) induce necrosis and are more cytotoxic, while NPs greater than 1.4 nm induce apoptosis (Pan et al., 2007). A study by Schaeublin et al. (2011) revealed that apoptotic cell death can be triggered by charged GNPs, while necrosis can be induced by neutral NPs. Exposure time and concentration are other parameters that could affect the triggering of necrosis. For example, in glioma cells, ROS-independent autophagic cell death is observed with low-dose NPs, whereas nano-C60 fullerene at high doses induces ROS-mediated necrosis (Harhaji et al., 2007).

9.5 OXIDATIVE STRESS AND INFLAMMATION

9.5.1 OXIDATIVE STRESS

Reactive nitrogen and oxygen species (RNS and ROS, and also jointly called RONS) are highly reactive derivatives of diverse processes of cell oxidation and are mostly identified by the occurrence of at least one unpaired electron in their outermost shells (free radical species). RONS are formed through a range of ecological (inorganic particles and radiation) (Martin et al., 1996) and biotic (peroxisomes, electron transport chain, and endogenous oxidase enzymes) origins (Brandes et al., 2010), and they are very essential in controlling natural cellular activities (Apel and Hirt, 2004).

Production of superoxide (O₂^{•-}) from the reaction of oxygen molecules is pivotal in the development of some RONS, specifically hydroxyl radicals (OH[•]), lipid peroxyl (LOO[•]), and peroxynitrite (ONOO⁻). Antioxidants, on the other hand, are compounds that equipoise the action of RONS. They function either directly by rendering inactive RONS or indirectly by decreasing the number of oxidized molecules. Also, they initiate the action of some other antioxidant compounds (Burke-Gaffney et al., 2005). In instances where protective antioxidant annuls RONS completely, it results in enhancement of bodily function, for example, reduction in the production of stress-related proteins and defensive antioxidant enzymes (Ji, 2001). Equally, a considerable imbalance between antioxidant and RONS can result in diverse physiological adverse effects in DNA, lipids, and proteins. Thus, this state is referred to as “oxidative stress,” which is said to be an important factor in the progression of aging especially in humans and a lot of diseases (Butterfield et al., 2006).

Production of adducts that serve as indicators for RONS-mediated reactivity is usually studied and quantified to know the levels of RONS in the body. This is due to reactive nature of RONS which makes them extremely difficult to measure or quantify directly (Harman, 2006).

Protein polypeptide chain backbone is reported to have a high susceptibility to RONS reaction (Stadtman and Berlett, 1997). Some amino acids like lysine, arginine, and histidine contain sulfur-hydryl (–SH) groups (which are redox-active), also referred to as thiol groups which consist of unpaired outer shell electrons

similar to some RONS. Thus, during the intensified phase of oxidative stress, the side chains of amino acid can react with some RONS like OH, thereby leading to oxidative alterations which stabilize the outermost shell of both molecules. Slightly oxidized proteins by the action of RONS are usually destroyed or made to undergo reduction through the action of proteasomes (Breusing et al., 2009). On the other hand, excessive protein oxidation can result in the accumulation of protein and the prevention of certain functions (Goto et al., 2007). This protein aggregate is detected with no trouble by the use of enzyme-linked immunosorbent assay (ELISA) (Buss et al., 1997) and spectrophotometric assays (Carty et al., 2000) or western blotting technology (Levine et al., 1994).

Nitration of protein occurs basically through the metabolism of nitric oxide by the production of some metabolites, which include nitrogen dioxide (NO_2) and peroxynitrite (ONOO^-) (Griffiths et al., 2006). Amino acids can be nitrated by the action of ONOO^- , and this is seen in its reaction with tyrosine which ultimately causes a conformational change (Souza et al., 2008). Various aspects of protein function can thus be regulated by this modification in proteins. Phosphorylations of tyrosine signaling and lipoprotein regulation are a few examples (Griffiths et al., 2006). Quantification of 3-NT is majorly done by the use of HPLC and electrochemical detection (also referred to as EC-HPLC), western blotting, etc.

Polyunsaturated fatty acids react easily with these free radicals (RONS). In plasma lipids and membrane, RONS could react to form stable products of lipid peroxidation (Quehenberger et al., 2010). Varying methods are usually employed in the quantification of these products of peroxidation, which include spectrophotometric methods, antibody technology, etc. (Breusing et al., 2010).

9.5.2 NANOPARTICLES, OXIDATIVE STRESS, AND INFLAMMATION

Inflammation and oxidative stress have been identified as the major toxicity mechanisms of NPs (Figure 9.2) (Khaled et al., 2019; Luyts et al., 2018). NPs could

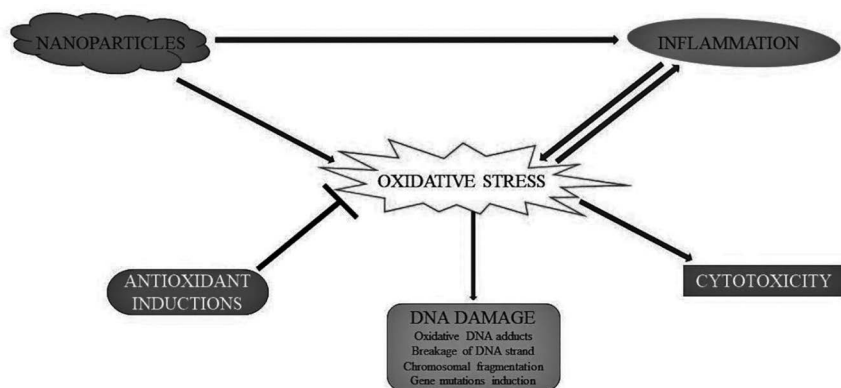


FIGURE 9.2 Mechanism of nanotoxicity.

facilitate the production of free radicals following exposure to ozone or atmospheric oxygen. These NPs-induced free radicals can disrupt metabolic processes and cellular structure. Free radicals produced by NPs react with macromolecules like DNA, lipid, and protein, thus leading to cellular damage (Luyts et al., 2018). According to Su et al. (2019), NPs could permeate bacteria cells, thereby generating a lot of free radicals resulting in oxidative stress and increased permeability of the bacterial cell membrane. The oxidative stress induced by NPs in bacteria helps reduce the population of antibiotic-resistant genes in the bacteria. Major causes of nanotoxicity have been attributed to the induction of reactive oxygen species (ROS) by NPs. These can be the result of NP–cell interactions and/or due to the presence of pro-oxidant functional groups on their reactive surface (Risom et al., 2005). ROS production is associated with processes such as cellular signaling and defense mechanism of the immune system. However, overproduction leads to severe damage to cellular macromolecules, thereby causing detrimental effects on cells.

In a separate study, cerium oxide NPs of various sizes (15, 30, and 45 nm) caused induction of oxidative stress (Eom and Choi, 2009). Also, AgNPs (4.7 and 42 nm) caused ROS induction, depletion of superoxide dismutase, and glutathione inhibition (Breusing et al., 2009). AuNPs with a size range from 5 to 250 nm led to higher amounts of cellular ROS (Misawa and Takahashi, 2011). Exposure to a single dose of silica NPs (*in vitro* and *in vivo* studies) leads to the induction of ROS, which in turn brings about pro-inflammatory responses (Park and Park, 2009). Furthermore, SWCNTs induced ROS generation, increased the level of lipid peroxide, decreased mitochondrial membrane potential, and decreased activities of enzymatic antioxidants (Wang et al., 2011).

In vivo studies of AgNPs using the fruit fly (*Drosophila*) model indicated induction of oxidative stress, DNA damage, apoptosis, and heat shock stress resulting in mediated developmental toxicity (Ahamed et al., 2010). Oxidative stress could release pro-inflammatory mediators through cascades such as the mitogen-activated protein kinase (MAPK), NF- κ B (nuclear factor- κ B), and phosphoinositide 3-kinase (PI3-K) pathways (Li et al., 2010). This suggests that inflammation is linked to oxidative stress reciprocally (Huang et al., 2010). Both *in vitro* and *in vivo* studies showed that NP-induced lung injury and pulmonary fibrosis lead to the ROS-mediated activation of NF- κ B and the production of pro-inflammatory mediators such as TNF- α , IL-8, IL-2, and IL-6 (Bryne and Baugh, 2008). Several metal oxide NPs including zinc, cadmium, silica, and iron have also been shown to exert their toxicity via the production of inflammatory cytokines induced by NF- κ B (Kahn et al., 2010; Wu et al., 2010).

Individuals exposed to NPs from electronics such as photocopying machine and printers have been reported to experience oxidative stress and inflammation of the respiratory tract (upper pharynx). This is because devices with toners emit high concentrations of NPs. Several invented NPs have been identified in toner-based devices. Photocopier users with greater levels of exposure to NPs from the machines had increased levels of several inflammatory cytokines (Khatri et al., 2017).

9.6 SAFETY OF NANOMEDICINE

Nanomaterials offer exciting opportunities in medicine. They can be used in various procedures including diagnosis, monitoring, control, prevention, and therapeutics. Nanomedicine has promising tendencies to revolutionize the field of medicine, though it has not been fully explored. More recently, nanomedicine is employed in targeted drug delivery to alleviate anticipated side effects, increase drug efficacy, increase dose at targeted sites, and reduce dose in other tissues. The tiny size of NPs may enable them to interact with various biological barriers in the body, and this may result in toxicity. On the other hand, this attribute may be exploited for drug delivery. However, it is very pertinent to understand if the nanoscale carriers exert any adverse effect. Therefore, it is important to determine the fate of nanomaterial upon diagnosis or therapy; and how the particles are excreted, biodegraded, or bioaccumulated; accumulation potentially leads to harmful long-term effects (Sally et al., 2014; Yamashita et al., 2012).

9.6.1 HAZARD AND RISK CHARACTERIZATION

The applications of nanotechnology are limited due to their inexpungible toxic tendency and detrimental effect, despite its vast popularity in medicine (Puja et al., 2015). The physicochemical properties of NPs, which enhance their application in nanomedicine, have also been incriminated in enhanced toxicological effects. Some of these properties are large surface area, small size, and flexible chemical composition. Of all properties, surface area and particle size are most important as these contribute directly and significantly to toxicity; smaller-sized NPs exhibit higher toxic effects due to their increased surface area (Puja et al., 2015). The structure and shape of NPs also influence nanotoxicity. The absorption of ions and biomolecules is determined by the surface of NPs, and this influences cellular responses, culminating in induced toxicity (Li et al., 2015). NPs have been at different instances found to exhibit molecular, cellular, immunologic, and tissue toxicities (Figure 9.3).

9.6.2 MOLECULAR TOXICITY

NPs immediately interact with cellular materials once they enter the body, due to the high free energy at the surface; they form a coat around the surface. The coat, also known as protein corona, has soft and hard layers. The soft layer is dynamic, while the hard layer is tightly associated with the NPs. The presence of protein corona changes the shape, size, and charge of the NPs. The formation of protein corona causes damage to circulating proteins (Wolfram et al., 2014). Some NPs have proven to induce structural alteration in proteins such as albumin, cytochrome, and ribonuclease. This ability to cause unfolding is attributed to the size. In addition, iron oxide injected intravenously could permanently cause iron transport damage by promoting the premature release of iron. NP-induced

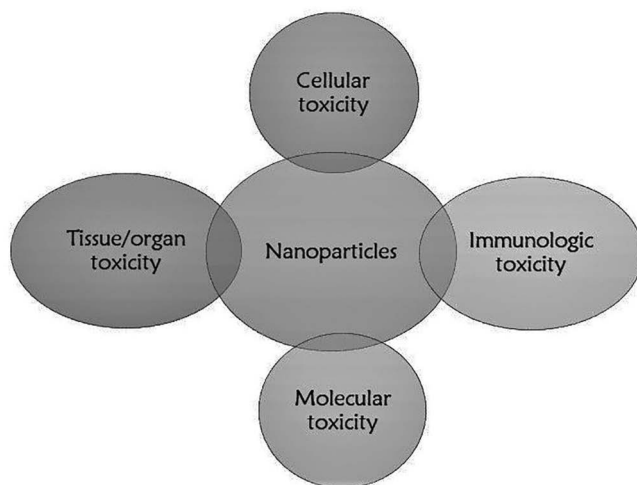


FIGURE 9.3 Different levels of nanotoxicity.

protein aggregation is another safety concern, where proteins clump together on the NP surface. This results in disease conditions such as the formation of amyloid (Wolfram et al., 2014). Also, endogenous biomolecules may be functionally and structurally altered when exposed to NP interface. These changes are important in the safety of NPs (Wolfram et al., 2014).

9.6.3 CELLULAR TOXICITY

Several NPs such as titanium dioxides, zinc oxide, polystyrene, and polycation particles have been shown to distort the lysosome membrane, resulting in the leakage of protons, iron, and hydrolytic enzymes. This causes protein aggregation, mitochondrial dysfunction, oxidative stress, and endoplasmic reticulum stress. Once the integrity of a cell membrane is interfered, the components of the cell leak out (Wolfram et al., 2014). NP-induced ROS can induce stress on cells, resulting in cellular damage. This in turn induces cells to develop cellular damage mechanisms such as apoptosis, regulated necrosis, and autophagic cell death (Xia et al., 2008).

9.6.4 TISSUE TOXICITY

Organs that have high levels of NP accumulation such as the liver are liable to tissue damage. Such damages may be traced to defects in the molecule and cell triggered by exposure to NPs. Studies have shown that lung inflammation occurred as a result of the deposition of CNTs in the lungs. Also, positively charged lipid NPs increased the presence of liver enzymes in the blood (hepatotoxicity) when injected to the blood (Muller et al., 2015).

Nanomaterials enter into the human body through the skin, orally, injection, or via inhalation. These particles affect the respiratory, circulatory, and central nervous systems as well as the gastrointestinal tract (Medina et al., 2007). A known adverse respiratory effect reported is a dose-dependent epithelioid granulomatous lesion in the lungs and persistent interstitial inflammation on chronic exposure caused by CNTs. Ceramic NPs induces oxidative stress/cytotoxic activity in the heart, lungs, and brain. These also have carcinogenic/teratogenicity effects. Moreover, nanomaterials injected cause secondary complications in the circulatory system and proceed to the central nervous system (Morimoto et al., 2013; Singh et al., 2014).

Single-walled nanotubes have been found to decrease cell adhesion and induce cell apoptosis by upregulating death genes or downregulating survival genes (Alazzam et al., 2010). If orally ingested, NPs translocate to the central nervous system and bioaccumulate. These particles cause axillary toxicity and brain damage due to disrupted neurotransmission (Shrivastava et al., 2014). TiO₂ NPs were studied in astrocytes of rats and found to inhibit cell survival rate, and they also caused edema, destruction of blood–brain barrier, and brain tissue necrosis. Nanomaterials get in contact with the dermal surface through cosmetic products and wound dressings. For example, Acticoat—a nanocrystalline silver-coated wound dressing—is used in burn treatment. Silver toxicity was reported in 30% of patients who used Acticoat (Trop et al., 2006). Broadly speaking, the accumulation of nanomaterials in organs and sites inhibits the application of NPs in nanomedicine, molecular diagnostics, and drug delivery systems.

9.7 ENVIRONMENTAL SAFETY OF NANOMEDICINE

Nanotechnology has found its way into medicine in form of nanomedicine. With the advent of nanomedicine, newer strategies are being explored for cancer, cardiovascular diseases, diabetes, and infection treatments (Lushchak et al., 2018). Several nanomaterials including dendrimers, emulsions, iron–polymer complexes, liposomes, micelles, and nanocrystals have been employed in the nano-drug delivery system which has been utilized as regenerative medicines, diagnostic tools, and therapeutic agents (Madhyastha et al., 2019). There are several regulatory bodies such as the European Medicines Agency (EMA), U.S. Environmental Protection Agency (EPA), and U.S. Food and Drug Administration (FDA) that ensure the safety of the nanomedicine administered to patients, but little attention is paid to their effects on the environment. Pharmaceutical products are found to affect the environment even at minimal concentrations, and this is also true for nanomaterials. When exposed to the environment as effluent or gaseous waste, they might impose certain threats to living organisms around.

Aquatic organisms can be affected when wastewater from the industry is carried into streams, rivers, and ponds as a result of accumulation of nanomaterials over a long period. The nanomaterials in water conglomerate and form larger particles which accumulate over a long time, and this can cause several modifications in aquatic organisms such as behavioral changes or eventually lead to

death. Wastewater released from the nanomedicine industry to the soil affects the plants that take up nutrient from the soil. Exposure of some nanomaterials such as silver NPs, which has strong antimicrobial potency, has caused damage to microorganisms in the environment which were not the main target (Yuan et al., 2019). Nanomaterials can be inhaled or absorbed into the body through the pores on the skin, thereby causing inflammation of the lungs and respiratory problems.

The effects nanomaterials have on the environment and human system might depend on:

- a. **Size of nanomedicine:** It is known that nanomaterials are smaller particles with larger surface area which makes them more reactive and they can easily penetrate cells which the larger particles cannot easily access.
- b. **Shape of nanomedicine:** There are several shapes of nanomaterials, including cubes, cylinders, rods, and spheres. For example, carbon-based nanomaterials such as SWCNTs are more toxic when compared to spherical fullerenes (Yuan et al., 2019). Also, different structures of nanomaterials have shown various levels of damage based on the type of cells (epithelial cells, lymphocytes, macrophages) involved.
- c. **Chemical composition of nanomedicine:** When the composition is altered by changing a metal used in the core, there will also be changes in the reactivity of the nanomaterial. Certain metals are toxic, and some are not toxic, but increasing their concentration may make them harmful. Therefore, the core should be well coated to avoid any damage that can be caused.

9.8 CONCLUSION

The advancement in nanotechnology has continued to attract huge attention probably due in part to the prospects it holds for diverse applications across the various fields of discipline. In this chapter, we have highlighted and discussed the toxicity/safety profiling of NPs/nanomaterials. Additionally, the various mechanisms underlying the toxicity of NPs/nanomaterials were extensively reviewed. Furthermore, the fate of these particles in the environment was mentioned and appraised. Together, this chapter serves to deepen our understanding of the biological effects of these NPs/nanomaterials.

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10 Insights into the Mechanisms of Nanotoxicity and Evaluation of Nanomaterials

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10.1 INTRODUCTION

Nanoparticles can be internalized into the human body through pulmonary route, gastrointestinal tract, skin absorption, and direct cell exposure (Duan et al. 2013). Toxicity, oxidative stress, DNA damage, and inflammation are the possible primary side effects of nanoparticles. Tissue and organ deposition, secondary toxicity, systemic inflammation, and altered functions are secondary side effects of nanoparticles (Fard et al. 2015). Toxicity of nanoparticles primarily depends upon the characteristics such as particle size, agglomerate size in suspension, size distribution range, elemental composition and contaminant levels, zeta potential, particle morphology, and density. Nanotoxicity also depends upon the dosage

metrics such as mass per volume (mg/mL), surface area (μm^3), particle number, single or multi-dose, and dose frequency (Braakhuis et al. 2014). Therapeutic nanoparticles can be delivered by inhalation, intratracheal instillation, injection, or ingestion (via the gastrointestinal tract). Nanoparticle entry into the human body involves systemic distribution (lymph and blood), crossing the internal cellular barriers such as blood–brain barrier, storage in the brain and fats, and transformation into other metabolites (Moinard-Checot et al. 2006). Nanoparticles may be eliminated from the human body to the environment through the skin, gill, and intestinal cells (Semmler-Behnke et al. 2007). Reactive oxygen species (ROS) are generated by endogenous sources such as electron transport chain, mitochondrial respiration, inflammatory response, microsomes, and peroxisomes (Nathan et al. 2013). Nanomaterials are reported to act as exogenous ROS inducers which lead to the toxic effects (Fu et al. 2014). An imbalance between the production of free radicals and the cellular antioxidant defense leads to oxidative stress (Pisoschi et al. 2015). In this condition, ROS react directly with cellular components which results in damage of cell molecules such as DNA, protein, and lipid. ROS can also interfere with cellular signaling pathways, which also leads to oxidative stress. Oxidative response results in unwanted cell responses such as oxidative damage to the molecular targets, uncontrolled proliferation, inflammation, and cell death by apoptosis or necrosis (Barzilai and Yamamoto 2004). Evaluation of nanotoxicity is important for the use of nanomaterials for therapeutic purpose and environmental protection. Cell uptake ability of the nanoparticles can be studied by scanning laser microscopy, electron microscopy, fluorescent labeling for cell organelles, optic microscopy, and fluorescence-activated cell sorting analysis (Behzadi et al. 2017). Chemical analysis of nanoparticles can be evaluated by electron energy loss spectroscopy, energy-filtered transmission electron microscopy, inductively coupled plasma atomic emission spectroscopy, energy-dispersive X-ray spectroscopy analysis, and mass spectrometry (Baer et al. 2010). Cytotoxicity and cell viability of nanoparticles can be evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays, lactate dehydrogenase assay, apoptosis/necrosis, BrdU assay, and terminal UTP nick end labeling method (Lewinski et al. 2008). Nanoparticle-induced oxidative stress and inflammation can be evaluated by glutathione reduction, superoxide anion, polymerase chain reaction (PCR) analysis, enzyme-linked immunosorbent assay, and western blot analysis (Tournebise et al. 2013). Nanoparticle-induced reactive oxygen generation can be evaluated by diacetate assay, Griess' test, PCR analysis, intracellular calcium, and antioxidant stimulation (Angelé-Martínez et al. 2017). Nanoparticle-mediated genotoxicity and DNA damage can be evaluated by Ames' test, micronuclei assay, comet assay, and PCR analysis (Golbamaki et al. 2015).

This chapter elucidates the toxic effects of different nanomaterials of therapeutic importance in various *in vitro* and *in vivo* models. As the small changes in the nanomaterials result in the difference in the properties, mechanisms of nanomaterials are discussed for individual nanomaterials. Evaluation methods of nanomaterial toxicity are summarized in the last part of this chapter.

10.2 NANOMATERIALS AND TOXICITY

Nanomaterial toxicity depends upon the size, shape, surface area, charge distribution, chemical reactivity, dosage, and exposure of nanomaterials. Nanomaterials act by different mechanisms depending upon their type and majorly enter into the cell by lipid oxidations which lead to damage of cell membrane. Nanomaterials from the environment enters into an organism by passing through barriers in the organism. Internalized nanomaterials are distributed, metabolized, and eliminated from the organism. The sequence of events are illustrated in Figure 10.1. Nanomaterials may cause genotoxicity and cytotoxicity by different mechanisms such as redox activity, ROS generation, inflammation activity, cation toxicity, photonic activity, and embryo hatching interference. Mechanistic injuries caused by various types of nanomaterials are summarized in Figure 10.2.

10.2.1 TOXICITY EVALUATION IN *IN VITRO* AND *IN VIVO* MODELS

The major limitation of nanomaterial toxicity evaluation is assessing the toxicity in living cells and animals. Determination of *in vitro* toxic concentrations is important for *in vivo* evaluation and subsequent selection of therapeutic doses. This section discusses the changes in cell activities upon exposure to nanomaterials at different concentrations. Treatment of human brain microvascular endothelial cells with aluminum oxide of 8–12 nm size at a concentration of 1–10 μM for 24 hours showed decreased cell viability and mitochondrial function, increased oxidative stress, and protein expression alteration in the blood–brain barrier (Chen et al. 2008). Treatment of human mesenchymal stem cells with aluminum oxide of 160 nm size at a concentration of 25–40 $\mu\text{g/mL}$ for 12 hours exhibited decreased cell viability (Alshatwi et al. 2012). Treatment of rat blood cells with aluminum oxide nanoparticles of 30–40 nm at a dose of 500–2000 mg/kg for

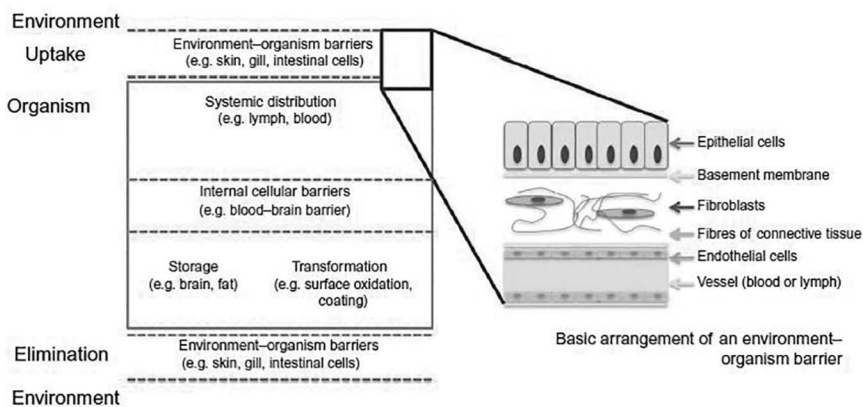


FIGURE 10.1 A diagram to illustrate entry and exit points of nanomaterials as well as internal processes. (Reproduced with permission from Schirmer 2014.)

Mechanistic Injury pathways

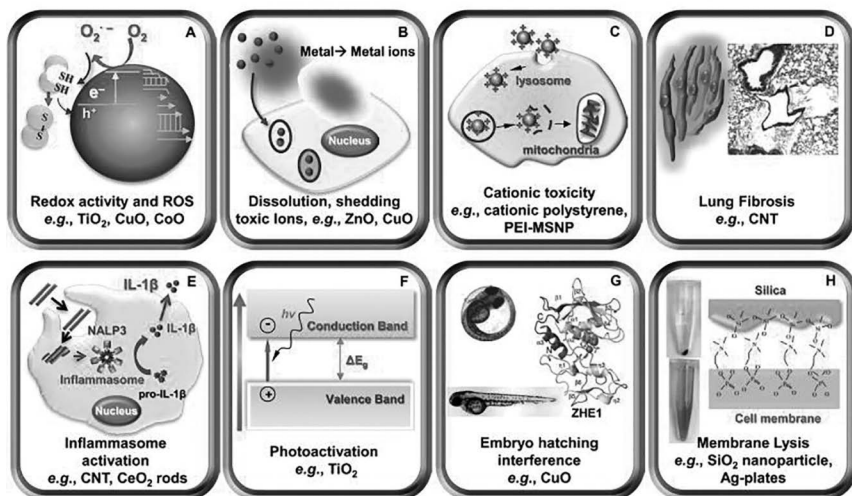


FIGURE 10.2 Various paradigms of mechanistic injury responses for high-throughput screening: (a) oxidative stress, (b) dissolution and release of toxic metal ions, (c) cationic injury to surface membrane and organelles, (d) pro-fibrogenic responses to CNT, (e) inflammasome activation by long aspect ratio materials, (f) photoactivation and influence of bandgap, (g) zebrafish embryo hatching interference, and (h) cell membrane lysis by surface reactivity. (Reproduced with permission from Nel et al. 2013.)

72 hours showed dose-dependent genotoxicity (Balasubramanyam et al. 2009). DNA damage was observed on mouse lymphoma cell line upon the treatment of aluminum oxide nanoparticles with 50 nm size at 5000 $\mu\text{g/mL}$ concentration (Kim et al. 2009).

Treatment of human lung epithelial cells with copper oxide nanoparticles of 50 nm size at concentrations of 10, 25, and 50 $\mu\text{g/mL}$ for 24 hours showed decreased lactate dehydrogenase, cell viability, and increased lipid peroxidation (Ahamed et al. 2010). In an experiment by Meng et al. (2007), nanocopper enters into mice and reacts with acid substance. It leads to alkalosis, accumulation of alkalescent substance, and copper ion overload in the gastric lumen (Figure 10.5). Treatment of lung cancer cells with multi-walled carbon nanotubes (MWCNTs) of 20 nm size at a concentration of 0.002–0.2 $\mu\text{g/mL}$ for four days decreased the cell viability (Magrez et al. 2006). Treatment of human alveolar carcinoma epithelial cell line with single-walled carbon nanotubes (SWCNTs) of 800 nm size at a concentration of 0–400 $\mu\text{g/mL}$ for ten days resulted in cell death (Herzog et al. 2007). Treatment of mice with SWCNTs of 10–30 nm size at concentrations of 40 and 200 μg and 1 mg for 90 days resulted in increased levels of lactate dehydrogenase (LDH), aspartate transaminase, and alanine transaminase (Yang et al. 2008). Treatment of Chinese hamster ovary cells, human epidermoid-like-carcinoma cells, and human embryonic kidney 293 cell lines with fullerenes of 178 nm size at a concentration

of 1 ng/mL for 80 days exhibited DNA strand breakage and chromosomal damage (Dhawan et al. 2006, Niwa and Iwai 2006). Treatment of human bronchoalveolar carcinoma cells with silica nanoparticles of 15–46 nm size at a concentration of 10–100 $\mu\text{g/mL}$ for 48 hours increased the levels of ROS and LDH malondialdehyde (Lin and Flint Beal 2006). Treatment of buffalo rat liver 3A cell lines with silver nanoparticles of 15–100 nm size at a concentration of 10–50 $\mu\text{g/mL}$ for 24 hours has shown decreased cell viability and increased LDH and ROS levels (Hussain et al. 2005). Treatment of human alveolar cell line with silver nanoparticles of 30–50 nm at a concentration of 0–20 $\mu\text{g/mL}$ for 24 hours reduced cell viability and increased ROS (Foldbjerg et al. 2011). Cell viability decreased upon the treatment of human leukemia cell lines with silver nanoparticles of 20–40 nm size (Haase et al. 2011). Fate and toxicity of nanosilver in biological and environmental media were summarized in Figure 10.3 (McShan et al. 2014).

Treatment of human colon carcinoma cells with zinc oxide nanoparticles of 50–70 nm size at a concentration of 11.5 $\mu\text{g/mL}$ exhibited increase in oxidative stress and decrease in cell viability (De Berardis et al. 2010). Treatment of human cervix carcinoma cell line (HEP-2) with zinc oxide nanoparticles of 307–419 nm size at a concentration of 10–100 $\mu\text{g/mL}$ for 24–48 hours resulted in DNA damage and reduced cell viability (Osman et al. 2010). In an *in vivo* experiment, mice treatment with zinc oxide nanoparticles of 30–70 nm size at a concentration of 14–20 $\mu\text{g/mL}$ for 12 hours resulted in decreased cell viability, DNA damage, and apoptosis (Sharma et al. 2012). In *in vitro* experiments on human hepatocytes, HEK 293 cell line treated with zinc oxide nanoparticles of 50 nm size and 100 $\mu\text{g/mL}$ concentration exhibited decreased cell viability, DNA damage, oxidative stress, and mitochondrial damage (Guan et al. 2012). Treatment of human bronchial epithelial cells with zinc oxide nanoparticles of size <20 nm at a concentration of 100 $\mu\text{g/mL}$ showed reduced cell viability and increased oxidative stress (Huang et al. 2010). Iron oxide nanoparticles of size 13–50 nm shown reduced cell viability on murine macrophage cells, human macrophages, human hepatocellular carcinoma cells, and rat mesenchymal stem cells (Jeng et al. 2006, Delcroix et al. 2009, Ge et al. 2009, Naqvi et al. 2010). Titanium oxide of 160 nm size with 1800 $\mu\text{g}/\text{mouse}$ resulted in DNA damage and genotoxicity (Liu et al. 2009). In an experiment on human lung cells treated with titanium oxide nanoparticles of <100 nm size at 10–50 $\mu\text{g/mL}$ concentration for 6–24 hours, results exhibited

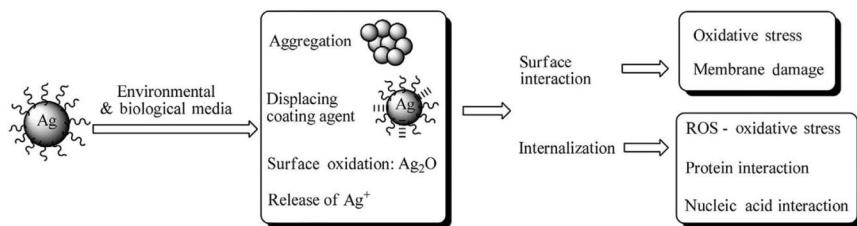


FIGURE 10.3 Schematic representation of the fate and toxicity of nanosilver. (Reproduced with permission from McShan et al. 2014.)

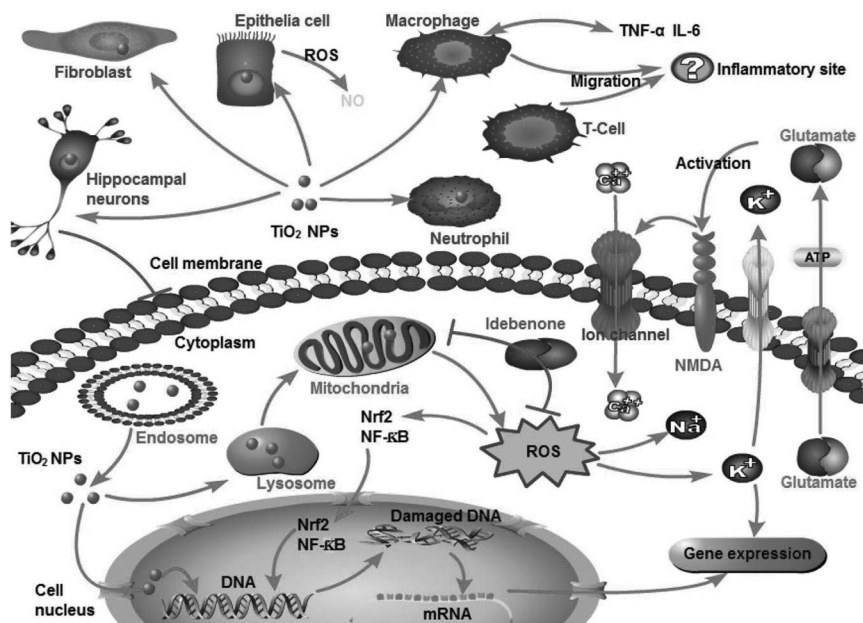


FIGURE 10.4 Schematic diagram of the toxicity of titanium dioxide nanoparticles (TiO₂ NPs) to invertebrates and vertebrates. (Reproduced with permission from Hou et al. 2019.)

increased oxidative stress and cytotoxicity (Bhattacharya et al. 2009). Schematic representation of the toxicity of titanium dioxide nanoparticles in invertebrates and vertebrates is illustrated in Figure 10.4 (Hou et al. 2019).

The biological assays for cytotoxicity evaluation are summarized in Table 10.1. Previous studies related to *in vitro* and *in vivo* evaluation of nanomaterials indicate that the exact nature of nanotoxicity is not elucidated yet. Nanotoxicity also depends upon the physicochemical characteristics of nanoparticles. Physicochemical characterization prior to *in vitro* and *in vivo* evolution is a prerequisite, and it helps design novel and safe nanomaterials.

10.2.2 DEVELOPMENTAL TOXICITY

Various developmental toxicities are reported upon exposure to nanomaterials. Early miscarriages, developmental retardation, reduced neonatal growth rate, and growth inhibition of embryos are some of the developmental toxicities observed in *in vivo* evaluation of nanomaterials. In an *in vivo* experiment, fetal length and neonatal growth rate were decreased upon exposure to cadmium oxide nanoparticles of 11 and 15 nm at a dose of 100 µg CdO/m³/2 days or 230 µg CdO/m³/day on 4.5 days (Blum et al. 2012). Intravenous injections of 10 ng, 100 ng, 300 ng, and 3 µg SWCNTs on CD-1 mice resulted in early miscarriages and fetal malformations (Pietroiuisti et al. 2011). Incubation of 25 or 50 µmol/L silver nanoparticles of 13 nm size on mice blastocysts resulted in apoptosis and developmental

TABLE 10.1

Summary of Mechanisms, Advantages and Disadvantages of Biological Tests Routinely Used for Cytotoxicity Evaluation. Reproduced with Permission from Moharamzadeh et al. (2009)

Biological Assay	Mechanism	Advantages	Disadvantages
MTT	Mitochondrial dehydrogenase activity	Rapid and inexpensive	Toxic to the cells
Alamar blue	Chemical reduction of culture medium	Accurate, and non-toxic fluorometric/colorimetric method	Expensive
Neutral red	Membrane damage (stains vital cells)	Non-toxic substance	Less accurate than Alamar blue
Propidium iodide	Membrane damage (stains dead cells)	It is possible to measure dead cells	Less accurate than Alamar blue
LDH	Cell damage	Simple assay, provides additional information when used with other assays	Poor dynamic range, lack of sensitivity
BrdU	Proliferation	Simple, rapid, and inexpensive	Less sensitive
³ H-thymidine	Proliferation	Rapid and sensitive	Radioactive assay
DNA measurement	Proliferation	Non-radioactive, sensitive, and robust	None
Protein content	Proliferation	Easy, rapid, and precise	None
Inflammatory markers	Inflammation indicators	Clinically relevant	Expensive and time-consuming tests
GSH	Toxicity indicator	Provides additional information about the toxicity of materials	Expensive and sophisticated
HSP	Stress indicator	Provides additional information about the toxicity of materials	Expensive and sophisticated
Apoptosis	Cell injury	Sensitive and specific for apoptotic cells	Expensive and requires specific equipment

retardation in blastocysts (Li et al. 2010). The number of apoptotic cells was increased, development of morulas into blastocysts was blocked, and blastocyst development was retarded upon the incubation of cadmium selenium (CdSe)-core quantum dots (QDs) of 3.5 nm at 125, 250, and 500 nmol/L for 24 hours on I mice blastocysts and morulas (Chan et al. 2008). Microinjection of amine-modified polystyrene nanobeads and carboxyl-modified polystyrene particles nanoparticles on mice blastocysts resulted in growth inhibition of embryos (Tian et al. 2009). Incubation of silica nanoparticles of 10 or 30 nm size at 1, 3, 10, 30, and 100 µg/mL concentrations for 24 hours or ten days on mouse embryonic stem cells resulted in inhibition of differentiation of stem cells (Park et al. 2009). Žalgevičienė et al. (2012) observed that intraperitoneal injection of CdSe/ZnS QDs and CdTe QDs

on the 6th, 13th, and 18th days of embryogenesis at 5 mg/kg did not cause any direct embryotoxic or teratogenic effects.

Physical and chemical characteristics of nanomaterials have a direct relation to developmental toxicity. Oxidative stress and inflammation may be part of the developmental toxicities. Detailed studies need to be conducted on a large scale to ensure the safety of nanomaterials.

10.2.3 NEUROTOXICITY

Some of the nanoscale materials can cross the blood–brain barriers and exhibit the detrimental effects on the nervous system. Nanomaterials can interact with glial cells and neurons, and this can lead to neurotoxicity in animals and humans. Moderate neurobehavioral alterations in offspring were observed on mice model upon the inhalation of titanium dioxide nanoparticles of size 97 nm at a dose of 42.4 mg UV-Titan/m³ 1 hour/day (Hougaard et al. 2010). Subcutaneous injection of anatase titanium dioxide nanopowder of 2570 nm size into mice at 100 µg/mouse/time resulted in alterations in the expression of genes related to brain development, central neural system function, and inflammation in offspring (Shimizu et al. 2009). Alterations in the cerebral cortex, olfactory bulb, and some regions related to dopamine systems were observed in mice model upon subcutaneous injection of anatase titanium dioxide nanoparticles of 25–70 nm size at a dose of 100 µg/mouse/time (Umezawa et al. 2012). In another study, subcutaneous injection of anatase titanium dioxide nanoparticles of 25–70 nm size on mice model resulted in apoptosis in the olfactory bulb of the brain (Takeda et al. 2009). Dopamine levels in the prefrontal cortex and neostriatum increased in ICR mice upon the treatment with anatase titanium dioxide nanoparticles of 25–70 nm size at a dose of subcutaneous injection at 0.1 mg/mouse/time (Takahashi et al. 2010). Oral administration of anatase titanium dioxide nanoparticles of <25 nm size at a dose of 100 mg/kg resulted in short- and long-term synaptic impairment of plasticity in rats (Gao et al. 2011). Downstream neuronal precursor genes and a patterning marker gene were reduced in expression upon the incubation of polyethylene nanoparticles of 33 nm size at a dose of 360 µg/mL for 48 hours on human embryonic stem cells (Hoelting et al. 2013). The molecular and cellular mechanism needs to be studied to evaluate the exact mechanism of neurotoxicity of nanomaterials.

10.2.4 REPRODUCTIVE TOXICITY

Potential male and female toxicity of nanomaterials has been observed to a certain level depending upon the physicochemical characteristics of nanomaterials. Reduced sperm counts, defective offspring, reduced infant growth, and hormonal imbalances are some the reproductive toxic effects of nanomaterials observed in animal models. Instillation of carbon black nanoparticles of 14 nm size at a dose of 0.2 mg/mouse resulted in seminiferous tubule vacuolation and reduced cellular adhesion of seminiferous epithelial cells (Yoshida et al. 2010). Decreased infant growth and disrupted development were observed on intraperitoneal injection

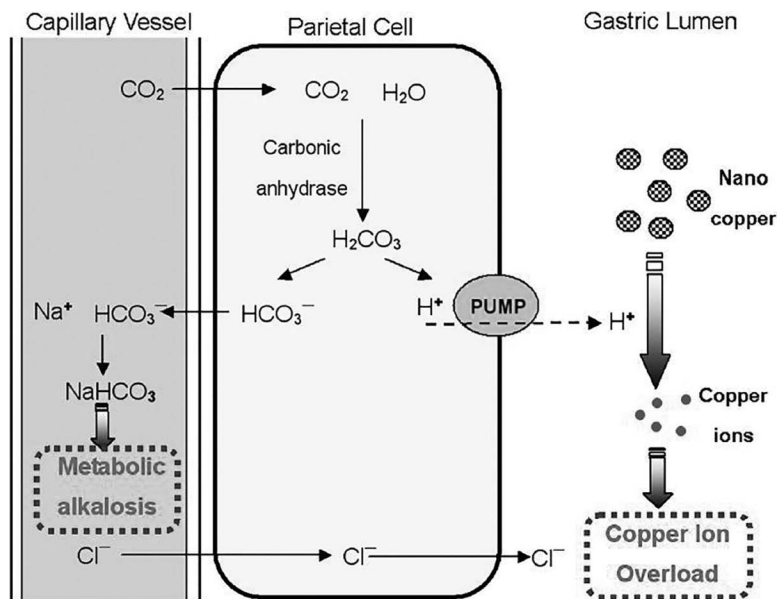


FIGURE 10.5 Schematic representation of copper ion overload in the gastric lumen. H⁺-K⁺-ATPase is indicated by an ellipse with “PUMP.” (Reproduced with permission from Meng et al. 2007.)

of dimercaptosuccinic acid-coated Fe_3O_4 nanoparticles of 3–9 nm size on mice model at 50, 100, 200, and 300 mg/kg doses (Noori et al. 2011). Inhalation of titanium dioxide (UV-Titan) nanoparticles of 17 nm size at a dose of 42 mg UV-Titan/ m^3 in an animal model resulted in changes in gene expression related to the retinoic acid signaling pathway in female offspring (Jackson et al. 2013). Inhalation and intratracheal instillation of 42 mg/ m^3 UV-Titan nanoparticles or 67 $\mu\text{g}/\text{animal}$ Printex 90 nanoparticles on mice models resulted in reduced sperm counts (Kyjovska et al. 2013). Subcutaneous injection anatase titanium dioxide nanoparticles of 25–70 nm size at a dose of 100 $\mu\text{g}/\text{mouse}/\text{time}$ resulted in reduced daily sperm production (Takeda et al. 2009). Nanoscale materials have exhibited reproductive toxicity in preliminary studies, and more studies are required to regulate the use of nanomaterials for human use (Figure 10.5).

10.2.5 TOXICITY IN ASTHMA MODELS

Inhalation of nanomaterials may increase the severity of respiratory diseases and lead to asthma. Intratracheal injection of carbon black nanoparticles on asthma mice model exhibited accelerated expression of IL-5 and activated Th2-like lymphocytes and eosinophilic inflammation (Inoue et al. 2005). Latex nanoparticles of 25, 50, and 100 nm size enhanced neutrophilic, but not eosinophilic, lung inflammation in a size-dependent manner on ovalbumin (OVA)-induced asthma mice models (Inoue et al. 2009a). Intra-nasal droplet application of titanium

dioxide nanoparticles of 250, 260, 29, and 14 nm size on OVA-induced asthma mice models exhibited increased lung-draining peribronchial lymph node cell numbers and production of OVA-specific Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) (De Haar et al. 2006).

In an experiment, intratracheal injection of 25 or 50 μg MWCNTs once a week for six weeks into asthma mice model exhibited allergen-induced airway inflammation and increase in the levels of T helper cytokine, chemokine, IgG1, and IgE (Inoue et al. 2009b). Inhalation of 100 mg/m^3 MWCNTs for 6 hours by OVA-induced asthma mice model showed elevated levels of platelet-derived growth factor (PDGF), transforming growth factor- β 1, and IL-5 mRNA (Ryman-Rasmussen et al. 2009). In a study, subcutaneous injection of MWCNTs into asthma mice models increased levels of IgE, IgG2a, TNF- α , eosinophils, Th2-associated cytokines, TNF- α , and neutrophil cells (Nygaard et al. 2009). In a study, *in vitro* and *in vivo* experiments were performed on SWCNTs and observed aggravated allergen-induced airway inflammation with mucus hyperplasia, and increased levels of OVA-specific IgG1 and IgE T helper cytokine (Inoue et al. 2010). In a study, intratracheal instillation of MWCNTs at a dose of 4 mg/kg once caused more obvious lung injury. They led to the formation of pulmonary fibrosis in rats with pre-existing inflammatory conditions (Cesta et al. 2010). Intratracheal instillation of SWCNTs and MWCNTs at a dose of 4 mg/kg on lipopolysaccharide (LPS)-induced asthma mice model enhanced LPS-stimulated expression of inflammatory cytokines and chemokines in lung tissue and circulation, including IL-1 β , macrophage inflammatory protein-1 α , and monocyte chemotactic protein-1 (Inoue et al. 2008). Intratracheal injection of 50 or 100 μg latex nanoparticles every week for six weeks into LPS-induced asthma mice model exhibited aggravated lung inflammation (Inoue et al. 2009a). Intratracheal instillation of TiO_2 nanoparticles and gold nanoparticles at a dose of 0.8 mg/kg on asthma mice model exhibited increased pulmonary inflammation and hyper airway reactivity (Hussain et al. 2011). Dose-dependent toxicity and time of exposure are also playing an important role in the respiratory toxicity of nanoparticles. Size of the particles can also regulate the inhalation and absorption of nanomaterials into the systemic circulation.

10.3 MAJOR PATHWAYS OF NANOTOXICITY

Extensive use of nanomaterials in various fields poses a threat to human health and the environment. Elucidation of nanotoxicity mechanisms helps regulate the use of nanomaterials across the globe. Nanotoxicity-associated oxidative stress leads to mitochondria dysfunctions, activation of immune cells, ROS toxicity, damage of organelles, inflammation, genotoxicity, and apoptosis (Risom et al. 2005, Xia et al. 2006, Limbach et al. 2007, Meng et al. 2009). Nanoparticle-mediated inflammation causes activation of toll-like receptors (TLRs) and NOD-like receptors (NLRs), uptake by immune cells, release of alarmins, NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome activation, and release of cytokines (Wang et al. 2015, Neagu et al. 2017). Nanoparticle-mediated

genotoxicity resulted in ROS accumulation, dissolution of toxic ions, inflammation chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts, and alterations in gene expression profiles (Singh et al. 2009, Magdolenova et al. 2014, Golbamaki et al. 2015). Nanoparticles-induced lysosome dysfunction leads to ROS toxicity, increase of lysosomal pH, disruption of lysosomal trafficking, NLRP3 inflammasome activation, release of ROS, ions, and hydrolytic enzymes, and apoptosis (Ma et al. 2011, Stern et al. 2012, Tahara et al. 2012). Nanoparticle-mediated mitochondria dysfunction causes depolarization of mitochondrial outer membrane, release of ROS, NLRP3 inflammasome activation, autophagy induction, and apoptosis (Lin et al. 2006, Pan et al. 2009, Khan et al. 2012, Coradeghini et al. 2013). Nanomaterial-mediated endoplasmic reticulum stress (ER) causes unfolded protein accumulation of ER, activation of ER stress signaling pathway, and autophagy to balance homeostasis and apoptosis (Tsai et al. 2011, Wang et al. 2012, Chen et al. 2013, 2014). Nanoparticle-mediated autophagy dysfunction causes blockage of autophagy reflex, excessive autophagy induction, and apoptotic and autophagic cell death (Stern et al. 2012, Panzarini and Dini 2014, Peynshaert et al. 2014).

10.4 TEST ASSAYS FOR NANOTOXICITY

Development of screening strategies for nanomaterials is the need of the hour, and various labs across the globe need to work together to maintain the regulatory guidelines. Sharing and analysis of the toxicity studies among the different parts of the world can ensure the safe and efficacious use of nanomaterials. Bacterial growth inhibition assay is a simple and rapid test showing whether the tested nanoparticles exhibit general toxicity to the medically important pathogenic bacteria and environmentally relevant bacterial strains such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* sp., and *Bacillus subtilis* (Bondarenko et al. 2016). *Vibrio fischeri* bioluminescence inhibition assay is a rapid and reliable standardized method for testing marine bacteria (Kurvet et al. 2011). Yeast (*Saccharomyces cerevisiae*) viability assay is the simplest model for eukaryotic cells, and algal (*Raphidocelis subcapitata*) growth inhibition assay is among the most sensitive ecotoxicological tests (Suppi et al. 2015). Environmentally relevant eukaryotic organisms can be tested by protozoan (*Tetrahymena thermophila*) viability assay (Jemec et al. 2016). Environmentally relevant freshwater organism can be tested by Crustacean (*Daphnia magna*) acute immobilization assay (Jemec et al. 2016) and Zebrafish (*Danio rerio*) embryotoxicity assay (Jemec et al. 2016). Environmentally relevant terrestrial organisms can be tested by Isopod (*Porcellio scaber*) cell membrane integrity assay (AO/EB) (Valant and Drobne 2012). Non-cancerous primary derived human cells can be tested by human mesenchymal stem cell membrane integrity (PI) and mitochondrial activity (MTT) assays (Zhang et al. 1999), and murine fibroblast BALB/c 3T3 membrane integrity and mitochondrial activity assays. Non-cancerous mammalian macrophages can be tested by rat alveolar macrophage NR8383 mitochondrial activity assay (Zou et al. 2014).

10.5 CONCLUSIONS

Commercial use of engineered nanomaterials for therapeutic purpose has increased in the past decade. Safety evaluation has gained importance for therapeutic implications of nanomaterials. Formulations with nanocarriers, formulation with active pharmaceutical ingredients, monoclonal antibody, recombinant enzymes, vaccines, and cytostatics are some of the classes of nanomedicines available in the market proved to be safe for therapeutic use. Toxicity evaluation of nanomaterials in *in vitro* and *in vivo* models presented in this study indicates that surface to volume ratio, chemical composition, surface charge, and accumulation of nanoparticles in the body contribute to their toxic effects. The dose is a key factor for safe and efficacious use of nanomaterials for therapeutic purposes. Nanotoxicology studies need to be advanced in collaboration with interdisciplinary approaches to evaluating accurate toxic effects of nanomaterials.

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11 Sensors to Monitor and Evaluate the Toxicity of Nanomedicine Applications

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11.1 INTRODUCTION

Advancement of nanotechnology, particularly its applications in medicines and pharmaceuticals, has revolutionized human life and health care in the twenty-first century. Application of nanotechnology in medicine includes medical substances that came from a multidisciplinary study that deals with the molecular structure developments and applications in dimensions less than 100nm called nanomedicines. In many cases the nanomedicine are often changeable with nanopharmaceutical (Bawa, 2015; Demetzos, 2016). The nanoscale properties of these medical

products make them suitable to create nanoproducts and devices such as therapeutic agents and other products of nanomedicine (Ge et al., 2014; Bawa, 2015; Demetzos, 2016). The biocompatible nanomaterials employed in medicines and pharmaceuticals are mainly targeted to improve drug delivery systems, imaging, and diagnostic technologies (Mansour et al., 2015). Therefore, nanomedicine has created breakthrough approaches in human health care and life in this era (Bawarski et al., 2017).

The first evolutionary milestone in the field of nanomedicine was the approval of Doxil® (liposomal doxorubicin) by the US FDA in 1995, which is followed by subsequent approval of other nanomedicine products (Demetzos, 2016). However, these approvals are in their early stage with less than 50 materials have been approved as nanomedicine drug formulations by the US FDA, while more others are in the pipeline (Weissig et al., 2014; Guzman-villanueva, 2015). Nanotechnology applications in medicines are greatly promising and attract many investors and companies to invest their money in research and development both in the pharmaceutical industry and the academia for the treatment of some of the most critical metabolic and genetic diseases such as genetic disorders and cancers as well as the development of effective drug delivery systems, imaging, and diagnostics (Weissig et al., 2014; Bawa, 2015; Guzman-villanueva, 2015). It is worth mentioning that nanomedicine provides humankind with great opportunities to improve their well-being and lifestyle. However, it also has limitations that should be managed thorough effective monitoring and imaging of these nanomaterials in the human body to ensure consistent drug safety and effectiveness that are approved by scientific and regulatory authorities (Bawa et al., 2016). One alternative approach to address this issue is rapidly using sensors to measure nanoscale properties and characteristics of nanomaterials used in nanomedicines. These sensors can be used as a platform for the creation of highly sensitive, selective, and quantitative devices for molecular diagnostics, monitoring, and imaging of nanomedicines. This chapter presents a review of literature on the sensors of nanomedicine and the possibility to use these sensors for monitoring the health risks associated with application of nanomedicines in humans. Thus, the emerging fields of sensors for nanomedicines are discussed, including characterizing of nanomedicines and the potentials of combining standard analytical techniques with sensors for nanomedicine detection and monitoring. Two kinds of sensors are discussed, namely, chemical sensor and biosensor that can be grouped into nanoscale-sized sensor called nanosensor and macro-/microscale-sized sensor. This chapter discussed that focusing the complex and crucial issues on sensors used for molecular diagnostics, monitoring and imaging of nanomedicines to evaluate nanotoxicity should be coupled with the development of novel methods of current instrumentation techniques.

11.2 NANOMEDICINES

Nanomaterials or nanostructured materials may have different characteristics due to differences in their chemical, magnetic, optical, and structural characteristics. For example, nanoparticles are smaller than prokaryotic and eukaryotic cells, and have a size similar to viruses (1–100 nm) (Shrivastava et al., 2007).

In the area of nanomedicine, where the word often changeable with nanopharmaceuticals, nanomedicine is described as the investigation of repair, construction, and control of biological systems in humans at the molecular level by employing nanostructures and nanodevices (Oberdorster et al., 2005; Bawa, 2015).

Generally, two important characteristics have been observed in nanomedicines (Mansour et al., 2015): drug solubility issues and drug targeted issues as given in Table 11.1. In drug solubility, these are often tackled by making nanoparticles. Here, pharmacokinetic profiles of different nanoparticles vary widely, since some synthesized nanoparticles could be soluble rapidly or but others very slowly. In the targeted issues, these are often overcome by making nanoparticles for targeted specific markers, cells, or locations in the human body. The physicochemical properties could increase the sensitivity of detection, improve the effectiveness of therapeutics, and decrease side effects (Shrivastava et al., 2007). On the contrary, these advantages of nanoparticle properties and characteristics could also increase side effects and other harmful effects (Oberdorster et al., 2005; Shrivastava et al., 2007). Furthermore, as a system, nanotechnology applications in the medical field could be briefly explained as shown schematically in Figure 11.1. Nanomedicine system is classified into two basic types, i.e. nanomaterials and nanodevices. These nanomaterials can be subclassified into nanocrystalline and nanostructured materials. Nanostructures include nanoparticles, dendrimers, micelles, drug conjugates, and metallic nanoparticles. Nanodevices can be classified into NEMS/MEMS, reciprocities, and microarray.

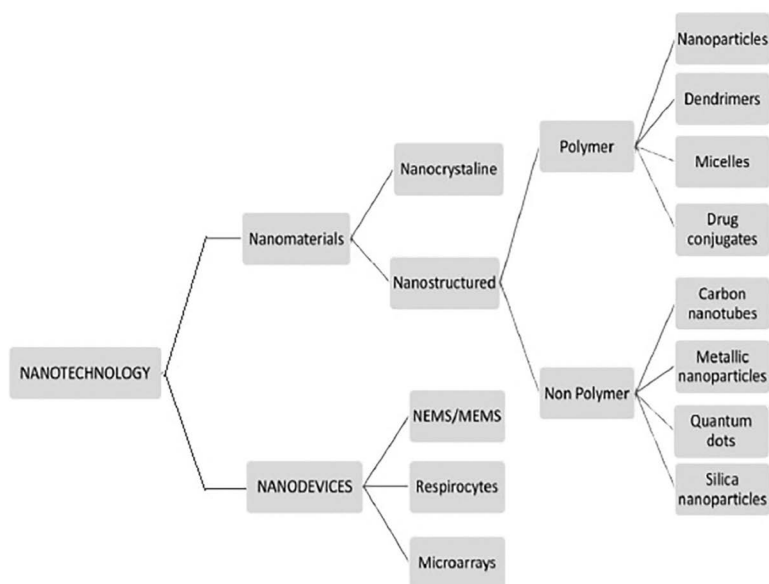


FIGURE 11.1 Schematic diagram of various types of nanotechnology in nanomedicine, that mainly divided into nanomaterials and nanodevices.

TABLE 11.1
Nanomaterials Used in Drug Delivery

Drug Delivery	Materials	Nanostructure
Biology	Lipids, peptides, polysaccharides, nucleic acids, viruses	Vesicles, nanoparticles, nanotubes, rings
Polymeric	Poly(lactic acid), poly(ethylene glycol), poly(glycolic acid), poly(ethylene oxide), poly(amidoamine), poly(L-glutamic acid), poly(alkylcyanoacrylate), poly(organophosphazene) etc.	Vesicles, nanoparticles, spheres, dendrimers, micelles
Carbon	Carbon, graphene	Nanotubes, fullerenes
Silicon	Silicone, silicone dioxide	Nanoparticles, nanoneedles, porous
Metal	Silver, gold, platinum, palladium	Nanoparticles, nanoshells

11.2.1 NANOMATERIALS AND NANOSTRUCTURES

The nanomaterials and nanostructures in medicines have attracted much attention owing to their promising features to improve and even more revolutionize drug delivery systems that include specific characteristics and useful compounds for both imaging and diagnostic agents (Figure 11.2) (Mansour et al., 2015). For example, liposomes, polymersomes, niosomes, micelles, nanostructured vaccines, dendrimers are nanostructured particulates that can be applied as useful agents in new and targeted drug delivery systems. These nanoparticles have specific structural and functional capabilities and properties, which serve specific applications if their size distribution, physical structure, composition, and components percentage are modified. In this context, nanoparticles could be reactive to the specific target, environment, or other analyte targets (Ogris & Oupicky, 2013; Weissig et al., 2014).

Liposomes have been extensively applied and most developed nanocarriers for novel and targeted drug delivery due to their small size (50–200 nm), biocompatibility, versatility, and good entrapment efficiency, particularly when dry phospholipids are hydrated and closed vesicles are created. They have been applied as long circulatory agents in passive and active delivery of gene, peptide, and protein (Mansour et al., 2015). Dendrimers are hyperbranched, and they have a treelike structure that consists of three different regions, i.e. core moiety, branching units, and closely packed surface. They have a globular structure and encloses internal cavities with the size less than 10 nm. They have been applied for a long time due to their size, shape, and unique physical characteristics (Mousa & Bharali, 2011). Carbon nanotubes (CNTs) are small macromolecules that are very important for biomedical applications. They have some special benefits compared to other drug delivery and diagnostic systems due to their unique physical characteristics (Fisher et al., 2012). Metallic nanoparticles have been applied in the drug delivery system, especially in the cancer treatment and in biosensors. Among them, gold and silver nanoparticles are important in circulatory delivery, bioactive material

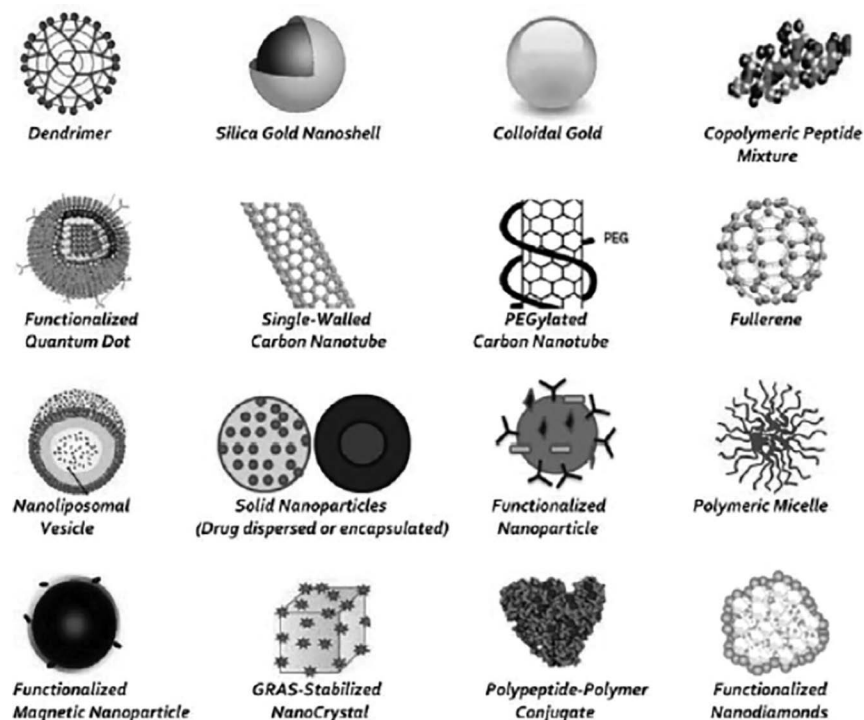


FIGURE 11.2 The main classes of nanomedicines that have been approved or in development (Mansour et al., 2015).

controlled delivery, bioactive particles targeted delivery to macrophages, and liver targeted delivery (Sheng et al., 2014).

Nanochips and nanoarrays are used as diagnostic and imaging agents in various methods to analyze various biomarkers in biological samples and monitor the formation of disease and its progress (Lee et al., 2009). Furthermore, nanotheranostics consist of both pharmacotherapeutic and diagnostic agents in one formulation with various purposes and targets. They could be used for drug delivery, drug release, drug efficacy, and monitoring of therapeutic drugs in one formulation. On the other hand, various nanoparticles have also been developed and applied to improve the diagnostic performances of imaging in nuclear magnetic resonance (NMR) (Abdelkader & Alany, 2012).

11.2.2 TOXICITY EVALUATION

Despite all the features of nanoparticles, there are still many concerns regarding nanoparticle safety when it is used in the human body (Saiyed et al., 2011). Toxicity evaluation in nanoparticles has received the most critical evaluation due to their exceptional properties such as size, size distribution, surface charge and characteristics,

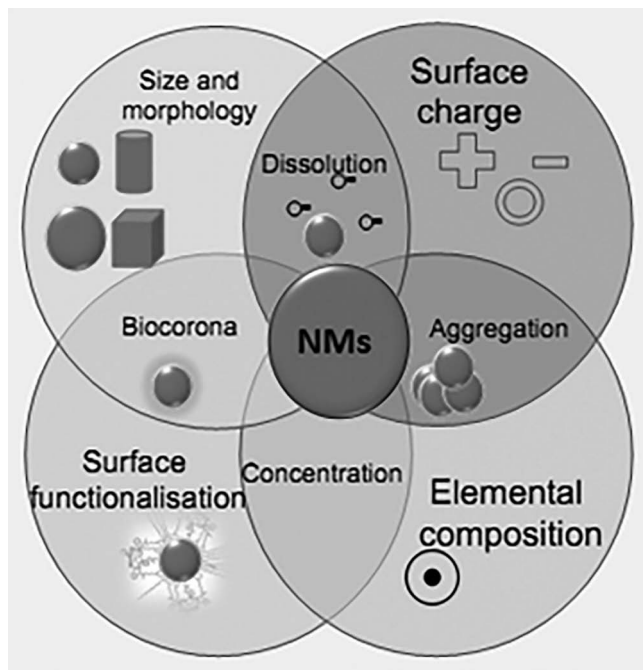


FIGURE 11.3 The number of possible causes of toxicity from nanomaterials due to their various physicochemical characteristics.

expanded surface area, stability, and self-assembly as shown in Figure 11.3 (Khanna et al., 2015). These features influence the characteristics of nanoparticles such as adsorption, distribution, metabolism, and excretion (ADME) that affect cellular uptake, body fluids distribution, and transport via biological barriers. For example, their nanosize allows them to cross the biological barriers such as in the eye, brain, or other cells. In this context, nanomedicines could trigger neurotoxicity, responses of inflammatory, or even the systemic circulation effects as they used in inhalation forms via their penetration into the central nervous system (CNS) via posterior nasal mucosal layer as it governs the toxicity profile to a lesser extent as an administration route (Zolnik & Sadrieh, 2009). However, our current knowledge is not enough to fully understand the toxicity mechanism of nanoparticles, because available toxicity tests are not fully assuring and the data received from *in vitro* and *in vivo* tests are not automatically extrapolated to human (Saeidnia et al., 2015).

Exposure to nanomedicines may not only affect the patients but also manufacturing workers, healthcare professionals, and others. Moreover, when they are disposed or excreted via wastewater, they may also be exposed to the public. Therefore, versatile detection and monitoring systems based on physicochemical properties are needed (Demetzos, 2016). Once the nanomaterials enter the human body, they may penetrate the epithelial and endothelial barriers, and then undergo cellular uptake processes like diffusion, and different endocytosis pathways like receptor-mediated

endocytosis dictated by their physicochemical and surface characteristics. Their biodistribution may also have relatively unknown patterns compared to other conventional pharmaceuticals, such as deposition on the CNS, and the growing fetus would be of high concern along with other organs like kidney, liver, and spleen.

On the other hand, cytotoxicity mechanisms of nanomedicines are not well known. However, oxidative stress, pro-inflammatory effects, and genotoxicity are possible through formation of reactive oxygen species (ROS), GSH/GSSG ratio alteration, transcription factors upregulation and signaling kinases, DNA damage, and mutation (Demetzos, 2016). Genotoxicity and cytotoxicity have been observed with nanodrugs containing metallic ions, such as metal oxides (aluminum, gold, silver, copper, titanium, zinc, and iron) and carbon-based nanomaterials (Saiyed et al., 2011). However, polymer- and silica-based nanomaterials have been raised to be relatively nontoxic and more biocompatible, as they may also involve cytotoxicity and ROS formation (Koopaei & Abdollahi, 2016). For example, some toxicity tests have been applied for nanomedicines before their approval both *in vitro* and *in vivo*. Validated *in vivo* tests should include at least ADME report, and acute and chronic organ toxicity tests; however, these *in vivo* tests are costly and time-consuming, and need ethical compromises (Shetab-Boushehri & Abdollahi, 2012). The OECD guidelines serve a wealth of technical documents for nanomaterials toxicity testing. However, to some extent, though this guideline for the toxicity tests on routine chemicals is useful, they need a more detailed discussion on the physicochemical characterizations and biohazards of nanomaterials used in medicines (Demetzos, 2016).

11.3 CONVENTIONAL METHODS FOR MONITORING AND IMAGING

A pharmacokinetic systematic and quantitative analysis, i.e. absorption, metabolism, distribution, and excretion, of the nanostructure can lead to improvements in the design of the nanostructure for therapeutic and diagnostic applications. In this context, the tremendously increased application of nanostructures in medicines has made the foundation of the emerging field of nano-monitoring and imaging. Therefore, increased attention needs to the study regarding the measurement and characterization of nanoscale properties to monitor and control nanomedicines to ensure consistent drug safety and therapeutic effectiveness. A significant study has been performed in this area (Saiyed et al., 2011; Bawa, 2015). In many studies, it is crucial to recognize that nanoparticle or agglomerate size distribution could change as a nanomaterial is produced. The nanomaterials interacting with a compound or an organism, in terms of size and shape, could change their original properties drastically (Bawarski et al., 2008). Hence, the doses, size distribution, composition, surface area, functionality, charge, and specification as well as porosity might differ from those when the nanomaterials were created or prepared.

Consequently, it is not easy to find one single test that can determine, measure, and monitor nanomaterials. Conventional techniques are only suitable for measuring physical and chemical parameters as given in Table 11.2. Therefore, these

techniques are suitable to be employed in conjunction with each other to obtain a better understanding of nanomaterials used in medicines. The current methodologies for detection, monitoring, and imaging of nanomedicines are briefly discussed including microscopy in conjunction with spectrometry, cell culture, and other techniques. As shown in Figure 11.4, there is an intersection between the monitoring and imaging techniques and characterization techniques of nanomaterials used in nanomedicines. Important characteristics that could be determined are particle size and distribution, shape, dose, purity, surface chemistry including charge, area, functionality, activity, specification, and porosity as well as the penetration ability in the biological systems (Bawarski et al., 2017).

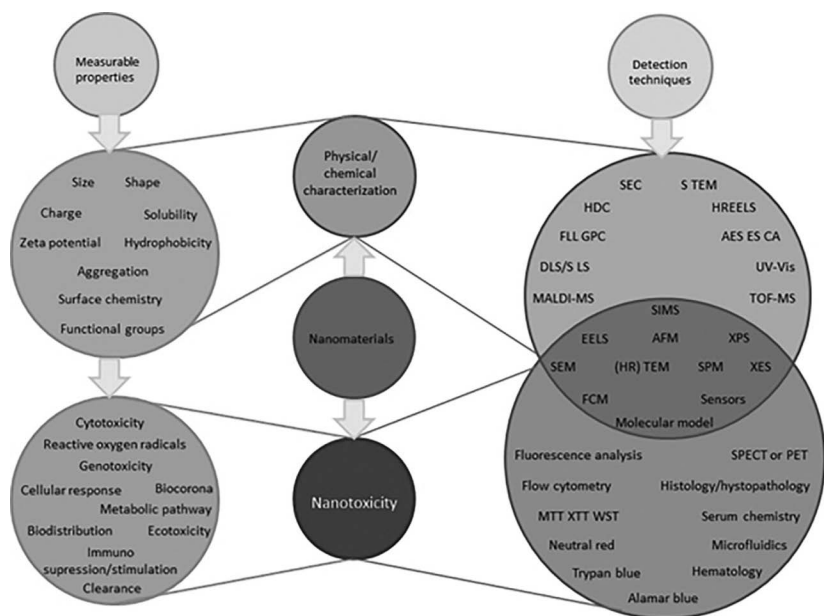


FIGURE 11.4 Major techniques employed for the characterization of nanomaterials used in nanomedicine, monitoring, and evaluation of nanotoxicity. Abbreviation key: SEM: scanning electron microscopy, TEM: transmission electron microscopy, SPrM: scanning proton microscopy, AFM: atomic force microscopy, AFFM: atomic force fluorescence microscope, GPC: gel permeation chromatography, SEC: size exclusion chromatography, SNUL: scanning near-field ultrasonic holography, GFAAS: graphite furnace atomic absorption spectrometry, NMR: nuclear magnetic resonance, XRD: X-ray diffraction, XPS: X-ray photon scattering spectroscopy, TGA: thermogravimetric analysis, FFF: field-flow fractionation, ICP-AES(OES): inductively coupled plasma atomic emission spectroscopy, TIRF: total internal reflectance fluorescence, EELS: electron energy loss spectroscopy, ICP-MS: inductively coupled plasma mass spectrometry, MALDI: matrix absorption laser desorption ionization, EDS: energy dispersive spectroscopy, PET: positron emission tomography, XPS: X-ray photoelectron spectroscopy, SRT: synchrotron radiation techniques, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test, XCT: X-ray computed tomography, WST: 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

11.3.1 MICROSCOPY

Microscopy is one of the most useful detection and imaging techniques in nanomaterials used for medicine. It is used to obtain information about nanomaterials such as their size, shape, and morphology. Examples of this technique are an electron and proton microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), scanning probe microscopy, scanning tunneling microscopy (STM), and atomic force microscopy (AFM), each of which has specialized performances. For example, SEM can be used to visualize nanomaterials in a scanning pattern by imaging the nanoparticle surface via scanning with a high-energy electron beam (Lin & Xing, 2008). While TEM has provided the most valuable information regarding *in vitro* nanoparticles study by allowing to find nanoparticles' location within a cell or tissue and characterize their composition before and after exposure to the cell or tissue, by coupling with spectroscopic methods (Warheit et al. 2007; Wu et al. 2008; Marquis et al. 2009). Especially, high-resolution TEM (HRTEM) could be the best selection to identify the nanoparticle crystalline structures (Warheit et al., 2007; Petri-Fink et al., 2008). However, its disadvantage is it requires special analysis conditions and special sample preparation, that is, it needs a high vacuum and very thin sample sections of limited particle diameter for the electron beam to penetrate across the sample. In addition, tissue sample preservation, fixation, and staining are required to preserve the sample details (Powers et al.,

TABLE 11.2

Conventional Techniques Generally Employed for Measuring the Physical and Chemical Parameters of Nanomedicines

Physical and Chemical Parameters	Analytical Techniques
Composition of element	AAS, FTIR, NMR, MS, ICP-MS
Size and morphology	TEM, SEM, AFM, FFF, HDC, HPLC, AUC, CLS disc centrifugation
Surface charge/functionalization	UV-Vis, AAS, FTIR, NMR, ICP-MS, XRD, HPLC, DLS GC/LC-MS
Biocorona	MS, SDS-PAGE
Dissolution	UV-Vis, ICP-MS, TFF
Concentration	UV-Vis, AAS, ICP-MS, HPLC, GC/LC-MS
Aggregation	UV-Vis, GC/LC-MS, HPLC, FFF, disc/gradient centrifugation

Abbreviations key: AAS: atomic absorption spectroscopy, FTIR: Fourier transform infrared spectroscopy, AFM: atomic force microscopy, NMR: nuclear magnetic resonance, UV-Vis: ultraviolet-visible, GC-MS: gas chromatography-mass spectrometry, HPLC: high-performance liquid chromatography, AOP: adverse outcome pathway, ICP-MS: inductively coupled plasma mass spectrometry, LC-MS: liquid chromatography-mass spectrometry, MS: mass spectrometry, BET: Brunauer Emmett Teller, DLS: dynamic light scattering, FFF: field flow fractionation, SEM: scanning electron microscopy, TEM: transmission electron microscopy, XRD: X-ray diffraction.

2005). In addition, scanning proton microscopy (SPrM) is an electron microscopy analogue that provide unique performance of high-accuracy elemental mapping down to the ppm level. However, the focusing proton technology is more complicated than for electrons, due to the fact that much protons higher momentum (Tong et al., 2008).

AFM is a combination of both electron microscopy and X-ray crystallography, and continuous developments in sample preparation, instrumentation, and imaging techniques have been made (Kada et al., 2008). It has been developed as an imaging method that could visualize biological sample structure in detail, e.g. proteins, DNA, cells, and membranes in their original environment and has been employed in various biomedical applications, such as the cellular toxicity tests of nanoparticles and CNTs (Kada et al., 2008). Furthermore, AFM is employed to determine the nanoparticle surface area that plays a crucial role in their properties and toxicity (Meng et al., 2010), including the nanoparticle shape and size (Chen et al., 2006). However, Engelhard, the multipoint Brunauer, Emmett, and Teller (BET) method is the most common method to measure the surface area. Here, the gas absorption to the particle surface is firstly determined, and then the surface area could be measured by the BET method. Transmission electron microscopy (TEM), scanning mobility particle sizer (SMPS), and diffusion charging (DC) are other methods that can be used for measurements of nanoparticles (Ku & Maynard, 2005). Atomic force fluorescence microscope (AFFM) has also been developed that has the capability for chemical(bio)-identification of optical methods, by combining with the high-resolution topographical imaging of AFM (Kassies et al., 2005). Scanning near-field ultrasonic holography related to scanning probe microscopy technique can be used to image nanoparticles buried below the surfaces of cells and could be powerful for nano-monitoring and imaging (Sahin, 2008).

11.3.2 CELL CULTURE

In vitro assays are the most used methods for animal testing, including assessment for chemicals cytotoxicity. Typically, it indicates the number of cells that are dead or alive after exposure to the chemicals tested. Classically, *in vitro* cell-based assays are generally employed to screen cytotoxic effects induced by chemicals in numerous cell systems. These assays include biochemical methods, e.g. neutral red uptake (NRU), lactate dehydrogenase (LDH), and ATP determination (Kroll et al., 2009), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) (Marquis et al., 2009), sulforhodamine B (SRB) assay (Papazisis et al., 1997) and WST (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Ngamwongsatit et al., 2008). Other assays include growth assays such as cytokine assay, phagocytosis assay, glutathione assay, nitric oxide assay, and colony-forming efficiency (CFE) assay (Papazisis et al., 1997; Ngamwongsatit et al., 2008). Thus, these techniques are useful for determination and measurement of the nanoparticles cytotoxicity.

Many reported publications have been focused on measurement of cytotoxicity of nanomaterials (Wise et al., 2007; Lewinski et al., 2008; Kirschner et al., 2015) by detecting the generated ROS and focusing on oxidative stress, mitochondrial perturbation, inflammation, lipid peroxidation, protein denaturation and degradation, and DNA damage (Nel et al., 2006; Sayes et al., 2006). These spectrophotometric methods can also be classified into tests that determine mitochondrial activity and that determine plasma membrane integrity. Furthermore, the cytotoxicity test can also be quantified using these methods, since spectrophotometric methods are the main instruments used in cytotoxicity assessment of nanomaterials (Lewinski et al., 2008; Marquis et al., 2009). However, it is important to understand nanoparticles' effects on mitochondrial activity, which is one of the many related cellular functions. The drawback of employing conventional assays is that nanoparticles may directly interfere with the signal transduction. This is due the improved reactivity of nanomaterial surface sites compared to bulk materials. For instance, silver nanoparticles were observed to directly interact with the tetrazolium salt of the MTT assay. Therefore, this shows greater than 100% viability of nanoparticle-exposed cells, due to the silver nanoparticles' ability to produce colored formazan product (Belyanskaya et al., 2007). In addition, SWCNTs' purity was able to convert MTT into its MTT-formazan insoluble form in the absence of any living system.

In medical applications, fluorescence imaging is one of the most optical imaging tools that currently received a lot of interest due to its high temporal resolution, non-invasive procedure, and relatively low cost. Fluorescence imaging in the visible region is commonly employed for conventional and intravital microscopy (Weissleder & Ntziachristos, 2003). It has been employed to measure the nanomaterial binding and uptake and also used for evaluation of the cytotoxicity of SWCNTs (Fiorito et al., 2006). Total internal reflectance fluorescence (TIRF) has excellent performance for fluorescent nanoparticles' real-time imaging for examining uptake and localization of dendrimer nanoparticles in gene delivery (Lee et al., 2007). Real-time NIR fluorescence imaging has been used for the measurement of quantum dots biodistribution (Pic et al., 2009). Two fluorescence dyes, i.e. calcein acetoxymethyl and ethidium homodimer, have been applied to test the viability of live cells exposed to nanomaterials, e.g. fullerenes and gold nanoshells. Alamar blue has also been applied for assaying cellular redox potential (Punshon et al., 2005) and has been observed to react with nanoporous silicon in the absence of cells (Low et al., 2006). Conducting multiple tests is beneficial to make sure that valid conclusions are made. *In vitro* cellular systems will require to be further developed, validated, and standardized relative to *in vivo* effects, in order to obtain valuable screening data regarding nanoparticles' relative toxicity in cells or tissues.

11.3.3 OTHERS

Microscopy and cell culture techniques could be used for the characterization of nanoparticle uptake and localization, biodistribution, and nanomedicines analysis, when our body has been exposed to nanomaterials. Several methods were focused

in this chapter including energy dispersive spectroscopy (EDS), inductively coupled plasma atomic emission spectroscopy/optical emission spectroscopy/mass spectrometry (ICP-AES/OES/MS), isotope labeling, and synchrotron radiation techniques.

Elemental analysis can also be used with a microanalysis system coupled with an electron microscope to determine the chemical composition of nanoparticles present in biological samples (Wick et al., 2007). For instance, EDS was employed to detect the silver nanoparticles present within cells (Powers et al., 2005), and electron energy loss spectroscopy (EELS) was employed coupled with TEM for elemental confirmation of CNTs uptake (Porter et al., 2007). ICP-AES/OES is commonly used as a useful tool for quantification of elemental nanoparticles (Szpunar, 2000). It is also used in *in vivo* pharmacokinetics study of quantum dots to measure quantum dots' distribution showing the mononuclear phagocyte system (MPS) uptake with no excretion, which, in turn, is a useful tool for tracking quantum dots *in vivo* (Fischer et al., 2006). ICP-MS was performed to explore how nanoparticles produce toxicity *in vivo* (Meng et al., 2007; Guo et al., 2008). The results show when the particle size decreases, for example in the case of copper, down to a nanoscale, it becomes extremely reactive in a simulative extracorporeal environment. In the nanoscale particle size, the large specific surface area will rapidly speed up the chemical reaction, which in turn may cause nanotoxicity, whereas the micro-scale compound does not occur. Thus, in this case, for example, the nanosized copper particles react with the hydrogen ions in the stomach much quicker than the micro-sized copper causing the massive production of Cu(II) ions that are highly toxic *in vivo* (Meng et al., 2007; Guo et al., 2008). In addition, carboxylated/oxidized diamond nanoparticles have been used to extract blood proteins, that has been centrifuged with the nanoparticles, mixed with a matrix absorption laser desorption ionization (MALDI) and performed with MS analysis using 2 orders of magnitude sensitivity improvement compared without nanoparticles (Kong et al., 2005).

Isotopic labeling is a tool for tracking a sample of substance passage through a system. The water-soluble hydroxylated carbon SWNTs have been labeled with radioactive ^{125}I atoms, then traced to study their distribution in mice, and show the number of CNTs accumulated in animal tissues (Wang et al., 2004). Two different nuclear imaging techniques have been employed to quantify quantum dots, including the positron emission tomography (PET) and single-photon emission computed tomography nuclear imaging (SPECT) (Dagar et al., 2003; Pic et al., 2009). In addition, other workers show an interesting fact that the fullerene derivatives' bioactivities were altered largely with the change of outer modified hydroxyl groups (Sayes et al., 2005). Since traditional tools such as X-ray photoelectron spectroscopy (XPS) and NMR were not good enough to measure the exact number of hydroxyl groups, a further quantification of the hydroxyl number was carried out using synchrotron radiation X-ray photoemission spectroscopy (SRT), where intensities for the hydroxylated and non-functionalized carbons were achieved (Chen et al., 2005). Moreover, magnetic resonance imaging (MRI) is an imaging diagnostic tool that can be used in the study of the pharmacokinetics of nanostructure, where the drug produced causes any observed toxic effect (Kobayashi et al., 2003).

A computer-assisted prediction approach, commonly called *in silico* method, can be used to determine the effects of nanoparticle physicochemical factors and predict the biological effects in various media as well as how they play an important role in toxicology in the systematic system (Kolanjiyil & Kleinstreuer, 2013). In the first attempt made to model the nanoparticle exposure to *in vitro* effects, aluminum (80nm) and its impacts on a rat alveolar macrophages population were studied (Wagner et al., 2007; Shelley et al., 2008). In many works, generally, the *in vivo* studies are applied to improve and validate the *in vitro* high-throughput screening (HTS) (Nel et al., 2013) and to build structure–activity relationships that enable to classified hazard ranking or toxicity level and modeling in silico by a suitable combination of *in vitro* and *in vivo* testing (Kato et al., 2009; Ruge et al., 2012). Thus, by combining technique using the classical approach for nanomonitoring and toxicity study, that involve in a silico modeling, a high-throughput screening, *in vitro* and *in vivo* testing regarding the potential effect and the toxicity of nanomaterials in the human body.

The most cytotoxic nanomaterials were also most effective at producing reactive oxygen species; *ex vivo* reactive species (RS) produced under UV illumination are associated with the biological response (Sayes et al., 2006). These data show that nano-titania samples optimized for RS, where by changing the hydroxyl groups number on the fullerene surface caused in a toxicity reduction by several orders of magnitude (Sayes et al., 2006). Thus, functionalization that often changes part of the entire nanomaterials' structure can highly affect their toxicity. Moreover, the fullerene potential toxicity was reduced significantly by employing these *in vitro* assays to the nanomaterials' chemical aspects that contribute to toxicity, showing that *in vitro* testing serves as a cost-effective and high-throughput *in vitro* assay that is suitable for the mechanistic models developed *in silico* to show which material should be developed (Sayes et al., 2005, 2007; Shinohara et al., 2009).

11.4 SENSORS FOR DETECTION NANOMEDICINES

Sensors, in particular chemical sensors and biosensors including nanosensors, represent a field of critical interest to determination, risk assessment, monitoring, and imaging of nanomedicines as well as molecular diagnostics. Here, chemical sensors and biosensors including nanochemical sensors and biosensors published in the literature related to the nanomedicines are discussed. This section provides a short overview of chemical sensors and biosensors including nanosensors for detection, monitoring, and imaging of nanomedicines.

11.4.1 CHEMICALS SENSORS

One important example of nanomedicines is silver nanoparticles (AgNPs) that generally are employed in diagnostic and therapeutic applications, aside from its antimicrobial activity. AgNPs' different formulations have been employed in biomedical applications, infertility studies, antibacterial effects, burns, skin damage, cancer treatment, etc. (Mathur et al., 2017). Due to their nanotoxicity, AgNPs have several disadvantages in various applications of therapeutics with possible toxicological

effects. For example, AgNPs have been synthesized with bovine serum albumin (BSA) and starch as capping agents to investigate their deleterious effects and distribution pattern in zebra fish embryos (*Danio rerio*) (Asharani et al., 2008). It was found that there was an increase in concentration-dependent mortality and hatching delay for the zebra fish embryos exposed to AgNPs (Asharani et al., 2008).

In the case of silver ions, there are many excellent chemical sensors used for detection purposes including ion-selective electrodes, optodes (Malcik et al., 1998; Kuswandi et al., 1999), and fluorescent sensors. Atomic absorption spectroscopy (AAS), plasma emission spectroscopy, and anodic stripping voltammetric methods have been employed to detect trace levels of silver (Howe et al., 2002). However, these sensors have been used rarely for the detection of AgNPs. One article has reported the application of these sensors for AgNPs, using selective chromogenic and fluorogenic probe for Ag^+ ions and AgNPs detection in the aqueous media (Chatterjee et al., 2009). The sensing mechanism was based on Rhodamine B derivative as the chromogenic and fluorogenic probe for Ag^+ /AgNPs in aqueous media. The probe forms colorless and non-fluorescent solution in ethanolic water (20% ethanol); when AgNPs is oxidized by hydrogen peroxide, Ag^+ ions were produced. The Ag^+ ion present leads to the pink color development at 558 nm and a strong orange fluorescence probe at 584 nm, indicating that the Ag^+ -promoted ring-opening eventually occurs (Chatterjee et al., 2009). The fluorescence intensity increased linearly with the AgNPs concentration. It shows that the probe can be used for the indirect determination of AgNPs, with a 14 ppb detection limit. This method is used to detect AgNPs in fabric softeners and sanitizer gel (Chatterjee et al., 2009). The disadvantage of using chemical sensors, including ion-selective electrodes and other sensors, is interference from other unwanted ions. These sensors are non-ion-specific, because other ions having similar properties can also be reacted.

Detection of AgNPs has been reported by using SPR since metallic nanoparticles plasmon resonances cause by the conduction electrons due to collective oscillation towards their matrix (Kreibig & Vollmer, 1995). Therefore, they can be used in determination of other metal nanoparticles such as gold nanoparticles (AuNPs). The detection and spectroscopy of AuNPs have also been reported employing supercontinuum white light confocal microscopy (Lindfors et al., 2004). This procedure also reported the first individual AuNPs detection below 10 nm. Another optical technique is the use of photothermal detection of gold nanoshells employing phase-sensitive optical coherence tomography (OCT) for detecting AuNPs (Adler et al., 2008). OCT is a high-resolution biomedical imaging technique that provides cross-sectional and three-dimensional images of tissue microstructure using interferometry, and it measures the amplitude and echo time delay of backscattered light (Huang et al., 1991). Typically, at low-temperature gradients, using OCT is useful for *in vivo* application and represents a novel method for detecting AuNP contrast agents with excellent signal-to-noise response at high speeds (Adler et al., 2008).

The fully imaging and spectroscopy method has been reported for individual AuNPs (Absil et al., 2008). It was based on a guided laser illumination with a

spatial modulation that is able to detect AuNPs at 10 nm. This method is excellent when employed with an imaging spectrometer and white incoherent illumination for the same system and delivered individual spectroscopy of several gold beads at the same time, allowing rapid and selective discrimination among individual metal nanoparticles, aggregates, or dust. Moreover, carbon-fiber microelectrode amperometry was applied to determine the chemical messenger molecules' dynamic secretion from nanoparticle-exposed cells. This method allows a specific molecular target detection based on applied potential, sub-millisecond time resolution, and endogenous concentrations quantitation of chemical messengers released during exocytosis (Huang et al., 2006; Marquis et al., 2009). In the AuNPs present, this amperometry was employed to characterize serotonin exocytosis from murine peritoneal mast cells co-cultured with fibroblasts, and the data obtained shown that AuNPs interrupt the dense-core biopolymer intergranular matrix and present the potential for systematic studies on how exocytotic function is affected by AuNPs composition, shape, and size (Marquis et al., 2009).

11.4.2 BIOSENSORS

Currently, there are various kinds of biosensors developed by incorporating bioelements, such as proteins, DNA, cells, and even tissues as bioreceptors, and various signal transducers devices, such as electrodes, optical transducers, microbalances, and semiconductors. These instruments have been employed in various applications, including nanomedicines. New kinds of protein biosensors are being developed by integrating an algorithm of novel protein engineering to generate site-directed mutants (Lambrianou et al., 2007).

Current technology has limited the dynamic and quantitative analysis of metabolites and signaling molecules for resulting temporally resolved information at the cellular level. Fluorescence resonance energy transfer (FRET) technology allows quantitative analysis of molecular dynamics in biophysics and molecular biology, e.g. the protein–protein interactions, protein–DNA interactions, and protein conformational changes monitoring (Okumoto et al., 2012). FRET biosensors have been used to monitor cellular dynamics at the single-cell level in real time and in heterogeneous cell populations that are suitable for applications in basic biology to biomedical and pharmacokinetic fields (Chaudhuri et al., 2011; Okumoto et al., 2012). Despite its various applications, FRET-based biosensors have many challenges. There is an increasing demand for improved specificity of FRET and higher fluorescence resolution biosensors (Chaudhuri et al., 2011). Furthermore, among many FRET-based technologies applied to medical diagnostics, the FRET reagents at low cost has become a crucial concern.

Genetically encoded FRET biosensors have been enabled to visualize numerous signaling events in living cells, e.g. protein phosphorylation and G protein activation (Miyawaki & Karasawa, 2007; Aoki et al., 2012; Chen et al., 2013). FRET biosensors have also been constructed to visualize the signaling molecules' activities in living cells, such as in cancer research (Aoki et al., 2012), that can be suitable for nanomedicine monitoring and measurements. A specific and sensitive FRET biosensor

has been constructed (Lu & Wang, 2010) and used to determine the BCR-ABL kinase activity in live cells. This biosensor enabled the cancerous and drug-resistant cell detection and the kinase inhibitor efficacy evaluation. Hence, this is a sign that future FRET biosensor development and imaging can significantly contribute to the diagnosis and therapeutics of cancer, including the monitoring of nanomedicines.

A cell-based assay system called real-time cell electronic sensor (RT-CES) system has been developed to monitor cellular events by determining the electronic impedance of sensor electrodes integrated on the bottom of microtiter (Solly et al., 2004; Lee et al., 2007). Cell index (CI) is derived based on impedance measurement, a dimensionless parameter, and served to provide quantitative data regarding the cells' biological status, such as cell number, morphology, viability, and cytoskeletal dynamics (Lee et al., 2007). A novel method with the RT-CES array has been developed to determine the combinative toxicity and specific toxicity induced by nanoparticles from the drugs or biomolecules that are attached to the medicinal particles (Roa et al., 2011). Here, potential harmful effects of nanomaterials, when they are applied in some medical situations, such as UV, ultrasonic, or X-ray, have also been studied.

11.5 NANOSENSORS

Besides the abovementioned chemical sensors and biosensors, there is another type of sensors that can be used to detect and monitor nanomedicines called nanosensors, which have the nanoscale size or nanoscale components or structures as shown in Figure 11.5 (Peng et al., 2009). Here, various kinds of nanoparticles are used to show different optical, fluorescence, and magnetic properties, and interactions between these properties make nanosensors highly potential for detection and monitoring of nanomedicines. The group of nanoscale sensors can produce a low-cost nanomaterials, power consumption, and reduced weight. This section highlights the use of nanosensors that can be classified into nanochemical sensors and nanobiosensors for sensing and monitoring of nanomedicines.

11.5.1 NANO-CHEMICAL SENSORS

The use of nanoscale materials, e.g. nanoparticles, nanotube, nanowire, nanosheet, nanocomposites, nanorod, nanoneedle, and nanobelt, for chemical sensing has seen increasing growth in the past decades. From a chemical sensing perspective, the small size of nanoparticles allows them to have large surface-to-volume ratios that bring to high sensitivity and rapid responses. Moreover, physical properties of nanoparticles, i.e. optical, electronic, and magnetic properties, can be fine-tuned via their size, composition, shape, and surface chemistry control, to create highly functional molecular probes (Wang and Yu, 2012). Surface-modified nanoparticles, e.g. AuNPs, AgNPs, QDs, MNPs, and CNTs, can have specific target-binding characteristics that enable highly sensitive and selective target detection. Different types of nanoparticles show different optical, fluorescence, and magnetic characteristics, and interactions between these characteristics

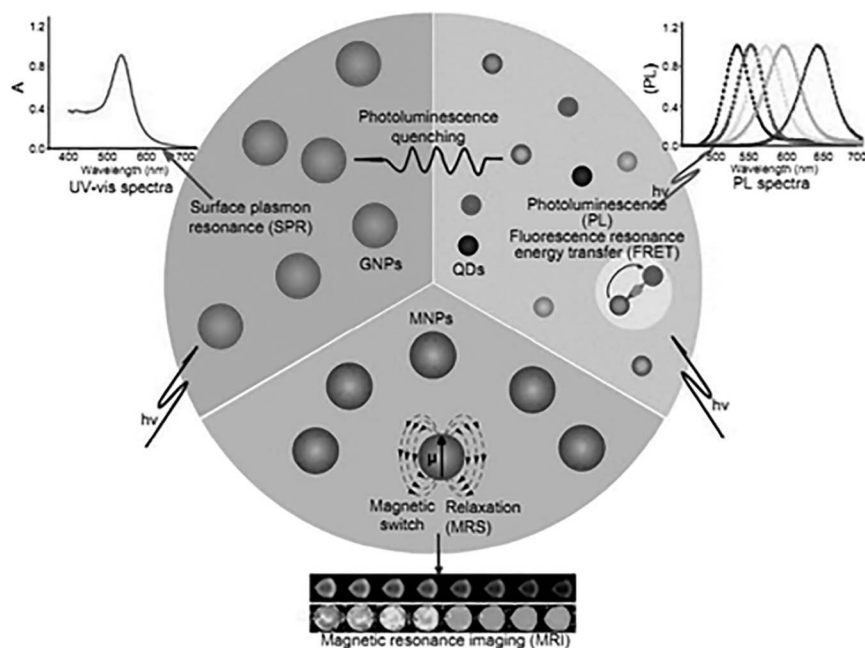


FIGURE 11.5 A schematic showing the properties of the nanoparticles used in nanosensors, such as gold nanoparticles (GNPs), quantum dots (QDs), and magnetic nanoparticles (MNPs) (Peng et al., 2009).

make nanoparticles highly potent for screening and monitoring nanomedicines (Mansour et al., 2015).

In terms of the application of nanoparticles as nanosensors for the detection of nanomedicines, there are more developments for nanobiosensors than nanochemical sensors as described in the next section. However, some of the works on the development of the nanochemical sensors of this area are described. Some of the scenarios are radio-labeled or magnetically or fluorescently tagged monitoring in biological matrices, such as lungs and numerous organs. NPs with radio-labeling specific radioisotope of one the contextual chemical elements of the particle-matrix became the gold standard for investigating kinetics of translocation, that allow real-time, efficient, highly sensitive, specific and selective, especially when they used to measure a balance of all distribution in the body and in excretions (Oberdorster et al., 1994; Kreyling et al., 1999). Furthermore, magnetic or fluorescent labeling of nanoparticles serves a powerful means of high specificity and sensitivity to determine translocated fractions in numerous target organs (Kreyling et al., 1999; Moller et al., 2001). Extreme care must be taken in order to make sure that every label attacks firmly with the nanoparticles; otherwise, they will be flawed severely. Moreover, the data evidence is needed that the labeling process does not modify the nanoparticles in its surface and function, owing to the surface is mainly interacting substrate with any biological systems.

In the case where nanoparticles labeling is not possible, tracking nanoparticles by electron microscopy as described previously may be a proper alternative to monitor the fate of electron-dense nanomedicines in the organism. The selection method for the study is a quantitative morphometric method that is by far preferable over qualitative spotting images, which may often make a wrong interpretation. Furthermore, it is strongly advised to find adequate alternate labeling techniques that may not be obvious at the first glance, but would still serve a reliable alternative, for instance, confocal microscopy used with fluorescently labeled nanoparticles. If the nanoparticle matrix has a property of chemical element or compound, chemical sensing of this property serves another strong alternative to track the fate of nanomedicines. In addition, contamination and possible endogenous background levels need careful distinction, and the lower level of sensitivity measurement of the chemical sensor need to be developed.

In the case of AuNPs, the advances in engineered AuNPs have generated tremendous reports and efforts on the emerging field of AuNPs plasmonics (Wang & Yu, 2013). Nowadays, advancement in creating novel nanosensors for chemical and biological sensing as well as imaging has been achieved (Durr et al., 2007). Thus, these sensors become promising tools for sensitive and selective analyses over traditional instruments, since they are suitable with portable devices that can be used in the field for nanomedicines on-site sample screening. Current research in this field is actively focusing on two lines, i.e. new materials, e.g. (i) anisotropic-shaped AuNPs, or gold alloy nanoparticles, are being prepared and new theories and models are being built and studied to increased our understanding of plasmonic phenomena at the nanoscale (Hicks et al., 2007), and (ii) new sensing instruments and/or platforms are being constructed and explored to achieve unprecedented resolution and sensitivity for nanosensing and imaging (Lee & El-Sayed, 2006). These researches are emerging interest to create next-generation nanosensors. However, it should be known that numerous critical issues still required to be solved before they can be used as a realistic tool for nanomedicines.

11.5.2 NANOBIOSENSORS

The development of portable nanotechnology-based sensors has great potential to fulfill the demands for high-throughput, rapid, ultrasensitive, low-cost bioassays for bio-monitoring chemicals and biomarkers (Barry et al., 2009). Nanobiosensors have been used in biomedicine (Prieto-Simón et al., 2008) and other applications (Ranjan et al., 2016). Since the report for NADH at low-potential detection by CNTs-modified electrodes (Musameh et al., 2002) and the AuNPs were first used as labels for electrochemical immunosensors (Dequaire et al., 2000). Many reviews are reported that partly deal with the nanomaterials used in nanobiosensors (Pumera et al., 2007; Pandey et al., 2008; Xiao & Li, 2008), and more reviews reported on CNTs-based sensors (Wang, 2005a) and metal oxide- and QDs-based biosensing can be found (Luo et al., 2006; Pingarrón et al., 2008).

Nanowires have also been reviewed as sensing materials (Yogeswaran & Chen, 2008). Based on their electrical, optical, and magnetic characteristics of these

nanomaterials, the biosensors based on these nanomaterials have been classified into electrochemical (Wang, 2005b), photo-electrochemical, optical, magnetic, mechanistic, and SPR-enhanced sensors. They are in the same size as biomolecules that unveil exciting possibilities for their interaction with biological species, e.g. tissues, cells, microbes, antibodies, DNA, and proteins. Extensive original research works and reviews based on nanomaterials for the electrochemical bioassays have been reported and are increasing continuously. They have been employed for construction of enzyme sensors, immunosensors, and DNA sensors to reach direct enzymes wiring and relative bioelements to electrode surface, to create spectroelectrochemical reaction, and to make barcode for biomaterials and signal amplification of biorecognition events.

The current progress in chemical detection and optical imaging developments using AuNPs and AgNPs has been reviewed (Murphy et al., 2008). The successful attachment of the functional group on the QDs has made it possible to improve the power of numerous molecular biological, biochemical, and physiological experiments, including tracking the movements of proteins within cells, the cell expression characterizing of determinant markers on the cell surface, and other works describing the utility in understanding the biological signal to exposures, potentially in whole organism investigations (Velasco-Garcia, 2009), and significantly reduces the use of toxic indicator and reference dyes to the cells (Xu et al., 2001). Nanomaterials provide unique characteristics that can be explored in nanosensors to detect nanomedicines (Bawarski et al., 2017). Nanobiosensors using an individual olfactory receptors are reported (Akimov et al., 2008) that mimic the way the noses of humans and animals respond to various odors and enable for rapid and noninvasive VOCs assessment, which can contribute to a metabolic state signature or diseases, that resulted in aromas of food, and be related with drugs, explosives, or pollutants.

Fullerene and CNTs detection using SPR biosensor has been developed (Kirschner et al., 2015). Fullerene and CNTs in aqueous solutions interact with proteins and bind to the surface, while their structures remain unchanged. The biosensor protocol basic is as follows. First, the gold surface is cleaned from macroscopic dirt particles. Next, streptavidin is attached to the gold sensor and a biotinylated C₆₀-fullerene derivative is also attached. The buffer solution is introduced before the sensor is primed to detect proteins binding to C₆₀. Proteins with fullerene affinity, monoclonal anti-C₆₀ antibodies, are passed across the surface causing a change in the binding index of refraction (Kirschner et al., 2015), which then corresponds to the number of fullerenes. The antibodies bind directly to the biotinylated, water-soluble fullerenes, and it shows that this biosensor can only specific to detect biotinylated fullerenes.

11.6 MICRO- AND NANOFUIDICS

Currently, the micro-fluidics progress is to develop micro- and nano-scale fluidic, where the fluidic paths mainly based on affinity reagents that can detect nanomedicines specifically. In the sensing scheme, there are two types of approaches, i.e. "off-chip approach" where macro-scale instrument is coupled, while the

“on-chip approach” consists of the micro-/nanosensing functions integration into microfluidic devices (Kuswandi et al., 2007). One example of this technology is microelectromechanical system (MEMS) devices. These devices are fabricated with similar microfabrication techniques as generally applied in integrated circuits (ICs). In biology, its application, known as BioMEMS, is growing fast in the biosensor and biomedicine areas, e.g. immunoisolation capsules, drug delivery, and pacemakers. The MEMS-based structure has been developed using nanoparticles manipulator, where nanoparticles could be injected selectively using thermal heating, mechanical vibrations, and electromagnetic waves. These works for the first time show the direct interaction control between nanoparticles and yeast cells in a liquid (Suzuki et al., 2016). The applications of microfluidics to cell culture-based tests are cell biochips, cell chips, micro-bioreactors (Sin et al., 2008; Wu et al., 2010). These cell-based microfluidic devices are becoming useful tools for rapid screening of drugs and chemicals (Wu et al., 2010). A microchip device integrated with DO sensors was constructed to test a substance within a matrix of different cell types and to study how a nanomaterial is targeted effectively to a certain organ or cell. Then, the detrimental effects of a nanomaterial have been studied on organs, e.g. kidney, heart, and liver (Sin et al., 2008). This technology will be very powerful not only to human and animal lives, but could also reduce resources, money, and time.

Nowadays, there are many studies carried out to construct sensor devices, with the aim to make automated platforms for the measurement and assessment of nanomaterials. Innovative and novel methods for sample introduction, fractionation, and passage to the sensor in various microfluidic platforms, such as labs-on-chips (LOCs) and micro-total analysis (μ TAS), have been developed to make these microdevices which can be used to monitor and assess nanomedicines. One of the promising technologies is organ-on-a-chip (OC) (Figure 11.6). OC is a microfluidic cell culture device produced using microchip fabrication methods,

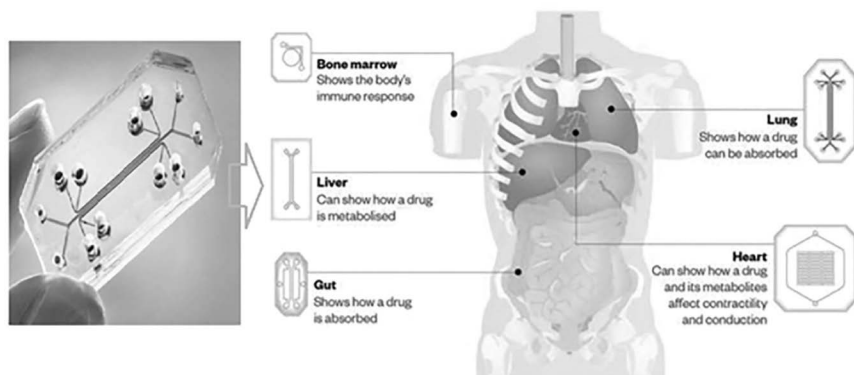


FIGURE 11.6 Typical organ-on-a-chip (OC) consisting of a microfluidic device that contains continuously perfused chambers inhabited by living cells arranged to simulate tissue- and organ-level physiology.

and it consists of continuously perfused chambers inhabited by living cells constructed to simulate tissue- and organ-level physiology (Bhatia & Ingber, 2014). By reconstructing the tissue–tissue interfaces, multicellular architectures, body vascular perfusion, and physicochemical microenvironment, this chip create tissue levels and functionality of organ that not enable with traditional culture systems of 2D or 3D (Wikswa et al., 2013). It consists of vital biomedical engineering elements similar to bio-MEMS. The LOCs and cell marriage have enabled the study of human physiology in targeted organs, employing *in vitro* multicellular human organisms in a new model. In the future, OC would be skipping the need for animals in drug research and development as well as its toxicity testing.

Although many works reported proving to have organ functions translated into this device, the development in OC for medicine application is still in an early stage. OC will vary in design and construction between various medical research settings. Therefore, optimization and validation of OC systems will need a long time and process. Here, organ functions have been translated onto OC devices, such as lung, heart, kidney, artery, cartilage, bone, and skin (Bhatia & Ingber, 2014). For example, a model as lung-on-a-chip for the human disease of drug toxicity-induced pulmonary edema has been developed (Huh et al., 2012). However, constructing reliable artificial organs needs accurate and precise cellular manipulation, as well as a deep understanding of the human body's signal to any toxic substance. General attention with OCs is in organ isolation during testing, otherwise, this chip could be useless. In the future, microfluidics, including micro-/nanofabrication and microelectronics, will offer promising sophisticated physiological responses in *in vitro* modeling under exactly simulated conditions that would be very helpful in nanomedicines research. Ultrasensitive detection and high-throughput technology based on nanosensors provide efficient and effective screening methods for many nanomedicine studies. Furthermore, this OC will find versatile and widespread applications in nanomedicine studies in the near future.

11.7 CONCLUSIONS AND FUTURE PERSPECTIVES

Besides the advantages of nanomedicines, they are also bringing a potential risk to human health, such as their ability to enter the human body and showing a biological activity that is related to their nanostructure. Recognizing and understanding the potential toxicity of nanomedicines in early-stage where less knowledge on the risks associated with nanomedicine development, modification, and applications becomes the key issue nowadays. Currently, numerous recommended nanomedicine testings show this progressing state of nanotoxicity. The sensing elements and screening strategy are obvious; however, the detailed technique will be developed and become more selective and focused as the results of this early nanotoxicity study become readily available. This chapter has presented the emerging field of sensors for nanomedicines toxicity evaluation, including the possibility of coupling current analytical techniques with conventional cytotoxicity methods. Traditional methods, such as microscopy and spectroscopy, for evaluating the properties of nanomedicines, mainly focus on the effects and

size distribution. In many cases, they are not compatible with the determination and measuring of complex matrices. Focusing on the complexity of potential toxicity of nanomedicines should be in-line with the development of new approaches and the development of devices and instrumentation.

Nanosensors have been described in detail, and particularly the use of nanomaterials for nano-chemical sensors and nanobiosensors is also reviewed. Chemical sensors and biosensors have also been described in detail. For example, there are many excellent chemical sensors for Ag^+ ions detection, such as ion-selective electrodes (ISE), optrodes, and fluorescent sensors. In addition, ICP-ES, AAS, and ASV have been applied to determine silver in trace levels. However, these methods have been used very rarely for AgNPs detection. Other potential sensor techniques have been described as reliable alternatives. They are easily operated, faster, and safer for detection, screening, and toxicity evaluation of nanomedicines in the first step.

This chapter also showed that there is rarely any single sensor or analytical tool that is currently available for detection, characterization, and monitoring of nanomedicines simultaneously. In this respect, the best approach can be integration of different methods to monitor and determine nanomedicines, especially in a complex system, like a biological system. In monitoring complex systems such as cells, bacteria, viral components, ash, pollens, and proteins, robust, portable, and low-cost macro-/microscale sensors are needed to distinguish between synthetic nanoparticles and naturally occurring nanoparticles, particularly with minimal sample preparation. In terms of the complex matrices, like biological matrices, another simple method is needed to prepare the natural particles as interferences by filtering all cellular substances that are bigger than the targeted nanomedicines. The ultimate target in employing sensors as devices for assessing and evaluating nanomedicines is to perform real-time comprehensive studies as well as a remote assessment of nanomedicine exposure. Reaching this target will need sensor advancements and sample treatment technologies as well as other supported technology, such as informatics, imaging, and remote sensing.

Although there are various methods available for the measurement of nanomedicines, there are critical gaps in knowledge about the side effects and risk assessment of nanomedicines. The nanomonitoring emerging field and development of efficient detection and measurement devices for tracking the exposure of nanomedicines from a various range of fabrication, formulation, and medical products. The approaches have been developed for natural or synthetic nanomedicines in simple matrices that could be optimized to serve the information needed, such as microscopy, chromatography, spectroscopy, as well as extraction, centrifugation and separation techniques of the sample. Moreover, integration of these devices with sample preparation techniques is needed for the detection and characterization of nanomedicines in living systems (Karen et al., 2008).

In vivo nanomedicine studies provide new opportunities, and monitoring scheme should enable measuring all of the vital parts of nanomedicines in tissues and organs, because many nanomedicines are synthesized with multiple elements. Precise and accurate measurement requires suitable sample preparation, such as the separation of nanomaterials from complex matrices. In addition, nanoparticle sampling is a

challenging task owing to several reasons, such as difficulty to find locations that represent accurately the real exposure based on the size and nature of the targeted particles. Nanoparticles separation can only be achieved at a high pressure drop, and collection of filtered nanoparticle samples for gravimetric analysis and characterization is only possible using high-volume sampling approaches (Sarnat et al., 2003). Thus, novel methods for isolation of nanomaterials are required. On the other hand, OC is a promising tool in translation tissue physiological functions, growing mammalian cell co-culture and artificial microenvironment constructed by microchannel networks (An et al., 2015), it has great potential to advance in the nanomedicine studies, including the study of pharmacokinetics, toxicity mechanisms, monitoring and toxicity testing of nanomedicines. Therefore, these devices were proposed to provide detailed information on nanomedicines exposed to human body. Further research is needed to develop novel methodologies and instrumentations.

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12 Systems Approaches for Toxicological Assessment of Nanomaterials

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12.1 INTRODUCTION TO SYSTEMS TOXICOLOGY

For centuries, toxicologists have relied on the analysis of gross phenotypes for explaining the impact of toxicant exposure. Such tests usually call for high chemical doses and only capture the effects when the damage is already done. To detach

from solely using apical endpoints, the 21st-century paradigm shift in toxicology brought about the idea of toxicity pathways, which are intracellular molecular events that diverge from the normal state in response to toxicant exposure (Bhattacharya et al. 2011). Not only do they describe the cellular processes that are perturbed, but they also inform on the mechanism of action by which a toxicant causes adverse outcomes (Kleensang et al. 2014).

These alternative test strategies also move toward reduction of *in vivo* experiments and implementation of the 3Rs (Replace, Reduce, and Refine) in animal testing (Russell, Burch, and Hume 1959; Accomasso, Cristallini, and Giachino 2018). While regulatory toxicology is heavily based on animal testing during the stage of non-clinical studies before human clinical trials, the translatability of such tests to the human situation has been strongly questioned (Bailey and Balls 2019). Luckily, current *in vitro* assay systems are very advanced, with the potential to eventually supersede animal testing. In addition to screening a large number of new compounds, *in vitro* assays provide mechanistic insights in human-derived test systems (e.g., pluripotent cell-derived cardiomyocytes) (Guth et al. 2019, Ribeiro et al. 2019). Finally, the combination of well-designed *in vitro* assays with toxicity pathways provides tools for estimating toxic doses in animals and humans based on dose–response extrapolation from extensive *in vitro* screening (Bhattacharya et al. 2011).

The systems toxicology assessment framework employs tools that go beyond standard toxicological endpoints, complementing them with high-throughput molecular readouts that can be further organized into biological pathways (Sturla et al. 2014). This systemic approach toward risk assessment offers a large extent of details that have the power to refine the molecular targets of toxicants along with the mechanistic knowledge of the paths that lead to these targets and, eventually, to adverse outcomes. In the following sections, we introduce the technologies and computational approaches that make systems toxicology such a powerful addition to traditional toxicity testing.

12.2 SYSTEMS TOXICOLOGY TECHNOLOGIES

12.2.1 HIGH-CONTENT SCREENING

High-content screening (HCS), also known as high-content analysis (HCA) or high-content imaging (HCI), is a method that allows simultaneous evaluation of multiple biochemical and morphological cellular parameters (Haney 2008). Its applications, ranging from simple biological research to drug discovery, help identify and characterize cellular phenotypic changes upon exposure to compounds, proteins, etc. (Taylor, Haskins, and Giuliano 2007, Haney 2008). HCS belongs to the so-called high-throughput screening (HTS) techniques, in which the effects of thousands of compounds on cellular phenotypes are simultaneously evaluated. In particular, phenotypic changes can involve increased or decreased expression of cellular proteins and/or changes in their compartmental distribution (Zanella, Lorens, and Link 2010).

The method relies on quantitative cellular analysis by combining imaging and computing methods. Overall, HCS involves two major processes, both generally automated: image acquisition and image analysis. The first process is enabled by an automated fluorescence microscope, which, similarly to standard plate readers, allows information acquisition in each individual well for different multiwell plate formats. Once images are acquired, cellular phenotypic data are extracted from each image by using image analysis software (Liszewski 2014).

HCS was originally developed for drug discovery with the intention to provide alternative secondary assays by using multiparameter and individual cell attributes. To some extent, HCS assays can also be considered toxicologically relevant because they measure different cellular physiological responses. For example, simple measures of cytotoxicity, such as cell number and shape, can be combined with more specific measures of organelle health, such as mitochondrial mass and membrane potential (Marescotti et al. 2016). Finally, similarly to flow cytometry, application of HCS multiparameter assays can help define subtle toxic states by cross-correlating multiple endpoints.

The method has improved with technological evolution, enabling increased throughput and ease of use. As a consequence, its application has expanded from traditional use in secondary screening to primary drug screening (Haney 2008). More recently, following the evolution of innovative cell culture systems, this method has further evolved to enable imaging and analysis of more physiologically relevant models, which are increasing in complexity owing to their development in the third dimension (Marescotti et al. 2019).

12.2.2 OMICS TECHNOLOGIES

Omics profiling refers to evaluation of large sets of biological molecules, such as the genome, transcriptome, epigenome, proteome, and metabolome (Hood and Tian 2012). Omics technologies are fundamental to systems toxicology, allowing simultaneous observation of thousands of molecular outcomes (Talikka et al. 2016).

12.2.2.1 Genomics and Epigenomics

The rapid development of DNA sequencing technologies in recent years has led to an explosion in the number of genome sequences available in public databases, with more than 55,000 such sequences being available today (<https://www.ncbi.nlm.nih.gov/genome/browse#!/overview/>). Third-generation DNA sequencing instruments have allowed us to obtain genomic information of unprecedented quality and quantity (terabytes per single run) as well as read lengths of more than 100,000 bases in one run. The ability to read the sequence of the whole chromosome is no longer out of reach (van Dijk et al. 2018). Functional genomics links this great abundance of genome sequence data with gene and protein expression information to extract knowledge on gene regulation in an integrative manner (Bunnik and Le Roch 2013).

Epigenomics is a subdiscipline of genomics that looks at the effects on gene expression and metabolism caused by factors other than modification of the

genome sequence. These effects could be due to modification of nucleotides by addition of chemical groups (such as a methyl group to a cytosine) or modification (acetylation, methylation, etc.) of the histones bound to the DNA sequence (Skvortsova, Iovino, and Bogdanovic 2018). Traditionally, epigenomics also includes miRNA regulation of gene expression (Saliminejad et al. 2019).

12.2.2.2 Transcriptomics

The transcriptome reflects the activity of the genome, and transcriptomic analysis is used to measure changes in RNA levels that mirror gene expression regulation at any given situation, including in response to toxicant exposures. Currently, two approaches are widely used in the field: chip-based microarray technology with a predefined set of gene probes attached to a solid substrate and a more recent RNA sequencing technology, which allows to capture all RNA sequences in the sample via high-throughput sequencing (Segundo-Val and Sanz-Lozano 2016, Lowe et al. 2017). While microarrays need a sequenced reference genome for probe design, RNA sequencing can also be used for organisms whose genome sequence is not yet available (Wang, Gerstein, and Snyder 2009). Rapidly evolving technologies allow increased measurement accuracy, high reproducibility, broad dynamic range, and high sensitivity (Wang, Gerstein, and Snyder 2009, McGettigan 2013). The single-cell resolution of profiling allows understanding of biological processes in unprecedented details in complex tissues and organs, in which different cell populations contribute uniquely to the response to both internal and outer environment cues. Currently, a number of single-cell sequencing technologies are available, and these are described in various review articles (Kanter and Kalisky 2015, Hwang, Lee, and Bang 2018, Chen, Ning, and Shi 2019, Kulkarni et al. 2019). The massive amounts of data generated by transcriptomic technologies are made available to scientific community through public databases. The first transcriptomics database to accept data from any source is the Gene Expression Omnibus (GEO), an international public repository for high-throughput gene expression data, now 20 years old (Clough and Barrett 2016). The omics study repositories are invaluable resources for systems biology applications that help integrate millions of data points for understanding the biology of entire systems. Overall, transcriptomic approaches provide greater coverage and are usually less costly than other omics assays.

12.2.2.3 Proteomics

While often the method of choice to investigate the global impact of exposures on a biological system, transcriptomic analysis does not explicate all the changes that can happen at the level of proteome (Vogel and Marcotte 2012b). Proteomics aims to quantify proteins present in a biological sample and is currently performed by using tandem mass spectrometry (MS/MS) approaches in combination with liquid chromatography (LC). Proteomics methods are divided into targeted and non-targeted methods (Altelaar, Munoz, and Heck 2013). Targeted methods are used to accurately quantify the concentration of a particular target protein or peptide (Shi et al. 2016), whereas non-targeted methods involve semi-quantitative

evaluation of the most abundant proteins, usually covering a set of no more than 3,000 proteins (Zhang et al. 2014). Experimental and computational approaches and the contribution of quantitative proteomics to systems toxicology have been extensively reviewed in Titz et al. (2014).

12.2.2.4 Metabolomics

Metabolomics is, in fact, a collection of numerous methods for quantifying diverse chemical classes of small molecules present in biological samples. It encompasses many thousands of metabolites, such as amino acids, carbohydrates, and lipids that are a part of normal or perturbed metabolic processes. To better separate the compound classes, gas or liquid chromatography columns of different polarities and other physical properties are used before the fractions reach tandem mass spectrometers (Koal and Deigner 2010). As in the case of proteomics, targeted or non-targeted methods are employed to accurately quantify specific molecules or for semi-quantitative screening, respectively (Titz et al. 2017).

12.3 MECHANISTIC UNDERSTANDING OF HIGH-DENSITY DATA

12.3.1 HCS DATA ANALYSIS

The processing and analysis of thousands of images acquired with HCS technologies calls for automated approaches (reviewed in Zingler and Heyse 2008), such as computational image analysis, for which several tools are now available as open-source resources (Usaj et al. 2016). The processing of these multiparametric readouts produces millions of data points, generating huge amounts of data. The ToxCast computational pipeline, tcpl, not only allows the storage of large amounts of data generated by HTS and HCS but can also be used for data normalization and dose–response modeling (Filer et al. 2017). GladiaTOX, an open-source solution for HCS data processing and reporting, was developed with the aim to complement the tcpl with additional features, such as the ability to generate structured analysis reports (Belcastro et al. 2019) (Figure 12.1). More recently,

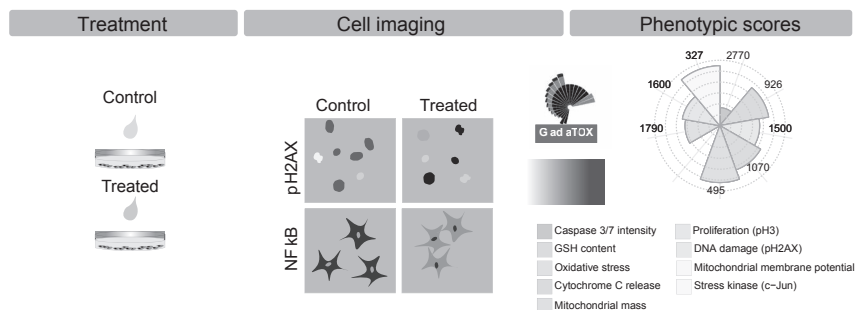


FIGURE 12.1 HCS workflow.

machine learning approaches have also emerged that employ HCS data for creating deep-learning networks (Kraus et al. 2017, Scheeder, Heigwer, and Boutros 2018, Smith et al. 2018), such as the extensive effort by Durr et al. that employed 40,000 publicly available images from treated samples to derive deep-learning classifiers for phenotype grouping (Dürr and Sick 2016).

12.3.2 OMICS DATA ANALYSIS

12.3.2.1 Pathway Analysis

There are several ways to obtain biological insights from omics data: A frequently used way is investigation of the biological function of molecules (genes, proteins, and metabolites) that are differentially regulated (up or down) in treated samples relative to the control sample.

Pathway analysis further aims to identify the molecular pathways that are affected by a treatment. Overrepresentation analysis (ORA) statistically maps the fraction of regulated molecules into known pathways; in essence, ORA evaluates whether a particular set of molecules in a pathway are represented more than expected by chance. There are several open-license pathway databases as well as analytic tools for toxicologists. Protein Analysis Through Evolutionary Relationships (PANTHER) allows users to upload their regulated genes as a list and performs enrichment analysis by using the Gene Ontology (GO) knowledgebase as the reference (Ashburner et al. 2000, Mi et al. 2019). The Kyoto Encyclopedia of Gene and Genomes (KEGG) (Kanehisa et al. 2013), BioCarta (Nishimura 2001), and Reactome (Joshi-Tope et al. 2005) collections of biological pathways can be accessed via tools such as the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics database (Huang, Sherman, and Lempicki 2008, Sherman and Lempicki 2009) (Figure 12.2).

The limitation of ORA is that it only takes into account statistically regulated molecules that fit the pathway. In contrast, gene-set enrichment analysis (GSEA) ranks the molecules on the basis of magnitude of change over the control and assigns them to *a priori* defined sets that have been grouped on the basis of their biological functions or coexpressions in previous experiments (Subramanian et al. 2005) (Figure 12.2).

While the above methods are extremely valuable, biological pathways are not just group of molecules; rather, they can be considered as graphs in which nodes are molecules, and edges represent interactions between the molecules. Therefore, methods that take into account the entire pathway structure, the various ways in which the nodes can be connected, and the number of reactions needed to connect two nodes in a given pathway provide an even better understanding of the biology that is impacted in the treated sample (Shojaie and Michailidis 2009, Khatri, Sirota, and Butte 2012). Such approaches allow to derive a pathway-level score, which tends to be more informative than a list of regulated molecules analyzed independent of the larger scheme of biology of which they are a part (Figure 12.2).

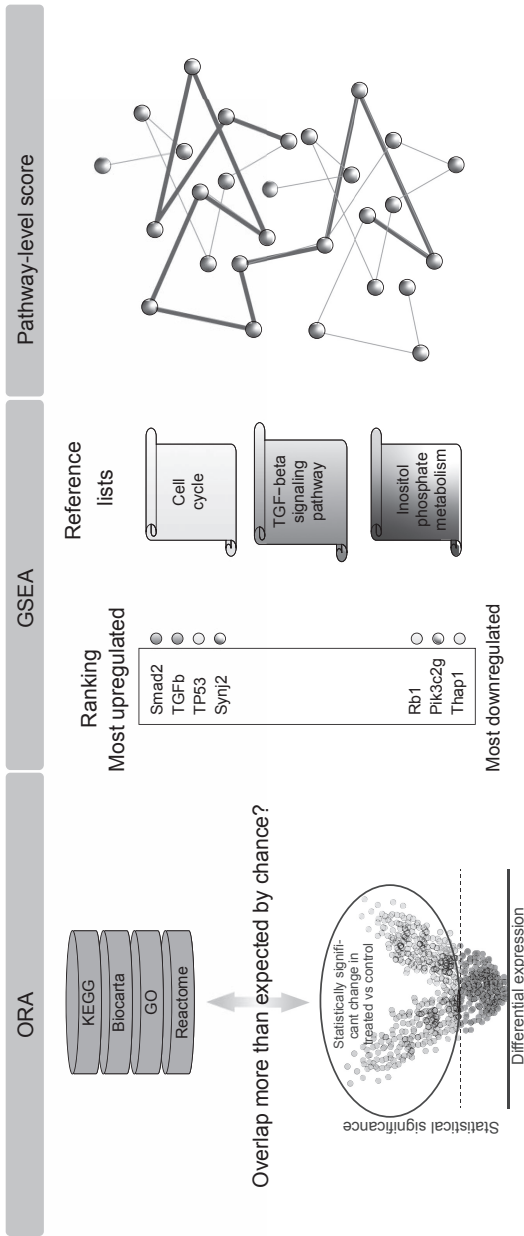


FIGURE 12.2 Pathway analysis of omics data. Overrepresentation analysis (ORA) uses statistical thresholds to map regulated molecules into known pathways. In gene-set enrichment analysis (GSEA), the molecules are ranked on the basis of their fold change in treated samples relative to the control samples. The balls indicate the *a priori* defined gene set to which each regulated molecule can be assigned. Pathway score is based on molecular changes and the dependence between the regulated molecules.

12.3.2.2 Biological Network Models

Systems biology and toxicology, by definition, studies biological systems at multiple levels, from molecules to cells to tissues and organisms, finally reaching a population-level understanding of the impact of exposure. Biological network models can be seen as an extension of individual signaling pathways, connecting several such pathways in the context of a biological process. Network models can be derived from scientific literature, omics data, or both (Raychaudhuri et al. 2003, Karlebach and Shamir 2008, Olsen et al. 2014, Hardt et al. 2018, Palma et al. 2018).

There are a number of ways in which literature-based knowledge can be leveraged to gain biological insights from high-throughput data. Causal biological network (CBN) models illustrate experimental findings from scientific literature manually curated in the Biological Expression Language (BEL) format (<https://bel.bio/>). BEL is used to capture causal and correlative relationships between biological entities, termed nodes, in computable statements (Figure 12.3). Distinct BEL statements then form complex networks within user-defined settings for signaling pathways, biological processes, diseases, xenobiotic exposure, etc. (Figure 12.3). Usually, to maintain each of these network models as a cohesive whole, gap analysis is performed, and additional relevant literature is identified on the basis of the gaps in the network models.

CBN models constitute a framework that allows integration and analysis of high-throughput datasets in a user-selected area of biology. Transcriptomics data are a valuable resource for linking literature-curated knowledge networks with mRNA abundances in relevant settings. The assumption that the abundance of an mRNA corresponds to the abundance of the respective protein holds true for as little as for 40% of transcripts (Vogel and Marcotte 2012a). Transcriptomic approaches fail to directly capture important molecular changes in the activities of factors acting upstream of mRNAs, because, often, the transcripts of these factors remain unchanged, while the proteins exert remarkable changes in their activity levels because of protein modification, translocation, complex formation, degradation, acquired stability, etc. However, transcriptomics data have the potential to estimate the changes in activity of regulating factors or even entire pathways by means of back-reasoning and deducing the activity levels of upstream factors through the changes in a pool of their dedicated transcripts (Catlett et al. 2013). Hence, the activity nodes in the model—such as transcription factors, enzymes, and microRNAs—are linked to the knowledge of their downstream gene abundance (downstream transcript layer) and referred to as inferable nodes (iNodes; Figure 12.3). iNode-supporting transcript content is generated by using transcriptomic datasets from publicly available studies where the iNode factor is manipulated in an experimental system, and mRNA pools that change their abundance in this condition are linked to the iNode for subsequent inference of the iNode factor activity level (Martin et al. 2012, 2019b).

Appropriate omics data from publicly available datasets are exploited by using the network perturbation amplitude (NPA) methodology (Martin et al. 2014, 2019a). At this stage, the power of the iNode-supporting mRNA layer is harnessed as the mRNAs detected in the omics study of interest are crosschecked against

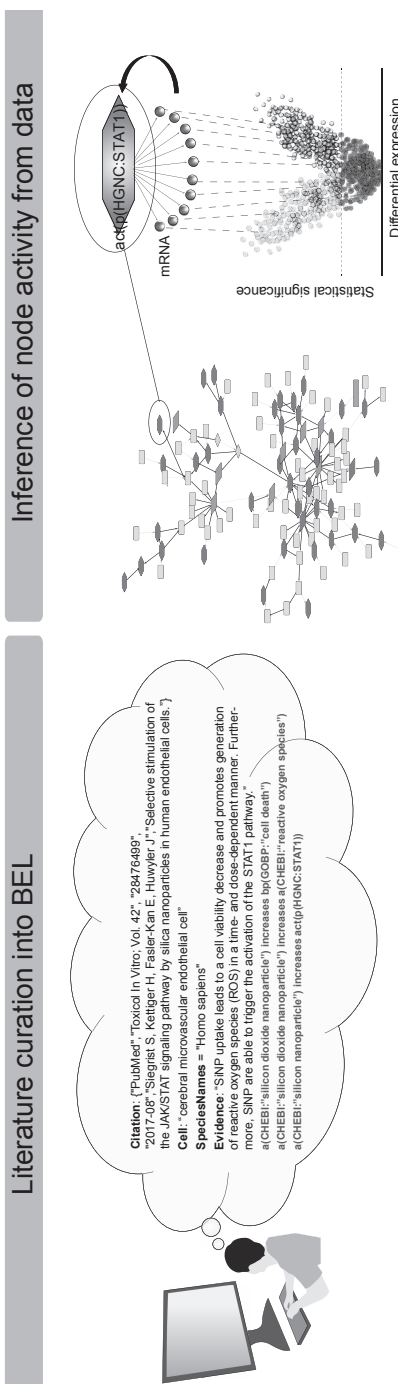


FIGURE 12.3 Causal biological network (CBN) construction and scoring. A literature search is conducted to find articles relevant to the chosen biological process. Causal statements are then converted to Biological Expression Language (BEL) to obtain computable biologic content. Next, all curated BEL statements are compiled into a graphical representation that offers a cohesive, structured, and browsable view of the biology. For data scoring, knowledge about mRNAs that are regulated by molded backbone nodes is used to infer the activity of that node from transcriptomic data.

the downstream transcript layer and used to infer the activity level of the node protein in the network model. NPA and subsequent network model analysis provide a powerful tool for transforming an unstructured bulk of dataset endpoints into scorable biological pathway perturbations in a variety of contexts. The highly connected nature of network models enables discovery of novel pathways within a user-defined biological framework. This approach opens up fresh perspectives for toxicity evaluation and can yield novel, and sometimes, unpredicted conclusions that can later be assessed with standard molecular biology techniques or traditional toxicological tests.

CBN models have proven to be a suitable basis for translating several thousands of data points into the context of known biological processes for gaining mechanistic understanding of high-throughput data (Iskandar et al. 2013, Park et al. 2013, Hoeng et al. 2014, Boué et al. 2015, Titz et al. 2016, Yepiskoposyan et al. 2019). Recently, we have also used this approach to reanalyze transcriptomic data from formaldehyde-exposed rat nasal epithelium. In the spirit of 21st-century toxicology, the original study conducted a gene expression benchmark-dose analysis to identify transcriptomic responses predictive of tumor formation two years after a 13-week exposure (Andersen et al. 2010). By using the network approach, our study provided a more detailed mechanistic insight into the dose effect with regard to carcinoma rate and identified a turning point at which two doses of formaldehyde inflicted opposite effects on biological pathways in a quantitative manner (Martin et al. 2019c).

12.4 SYSTEMS TOXICOLOGY FOR NANOMEDICINE

Technologies on a nanometer scale are a major step forward in the technological revolution of the 21st century (Wennersten 2008). Medical application of nanotechnology is at the core of nanomedicine, a relatively new branch of medicine. Nanomaterials, nanodevices, and nanoscale toolsets are being progressively used in the areas of drug delivery, diagnostics, and disease prevention and treatment. Nanoparticle-mediated drug delivery approaches actively target diseased cells and, therefore, offer remarkable advantages such as reducing the overall consumption and side effects of drugs. By functionalizing their surfaces, nanoparticles can be tailored for specific applications such as sensing certain biomolecules and cells or crossing the blood–brain barrier for improved targeting of the brain (Mout et al. 2012). Application of the nanoscale materials in diagnostics allows early and noninvasive detection of diseases (Sonali et al. 2018). Theranostics, yet another emerging discipline, provides the benefits of drug delivery and diagnostics by harnessing therapeutic and imaging nanotechnologies in a single nanovector for real-time visualization of drug delivery, drug release, and treatment efficiency (Chen, Gambhir, and Cheon 2011).

Nanomedicine offers unique opportunities in precision medicine and stands out from the traditional one-size-fits-all approach (Fornaguera and Garcia-Celma 2017). However, the nanoscale size poses new challenges in terms of potential toxicity of the engineered nanomaterials. Often, nanoparticle-associated toxicity is not related to the bulk chemical they are composed of; rather, it depends on

the particle size, shape, and surface features (Buzea, Pacheco, and Robbie 2007, Singh 2016). Assessing the risk of increasingly sophisticated nanomaterials is the focus of nanotoxicology, a research field aimed at determining whether and to what extent nanoparticles might pose a threat to biological systems and the environment (Maynard, Warheit, and Philbert 2011). In view of the novel and complex characteristics of nanomaterials, new testing methods are needed for time- and cost-effective, ethically acceptable toxicity evaluation (EPA 2007). Development of new nanotoxicological assessment methods that address the challenges of medicine on a nanoscale would also assist regulatory bodies and authorities in elaborating nano-specific regulatory guidelines (Rauscher, Rasmussen, and Sokull-Klüttgen 2017).

The chemical composition, size, shape, and surface characteristics of nanomaterials vary greatly, giving rise to an immense number of variables, which makes precision targeting challenging. The use of traditional animal toxicity studies for risk assessment of nanomaterials on such an extensive scale is unrealistic from time, cost, and ethical perspectives (Halappanavar et al. 2018). In a recently proposed hazard evaluation strategy concept, Siegrist and colleagues outlined a 3-tier framework—covering physicochemical characterization of injected nanoparticles, nanoparticle (bio)interactions, and hazard assessment—that facilitates the discovery of toxicity mechanisms by grouping nanomaterials by their mechanistic fingerprints (Siegrist et al. 2019). After passing tiers 1 and 2, suitable nanomedicines are assigned to different risk categories, which dictate the choice of assays for further hazard assessment in tier 3. Along with existing hazard assessment assays, exciting new possibilities are open for implementation in tier 3 by leveraging systems toxicology methodologies and omics technologies.

HCS-based toxicity evaluation can complement the existing knowledge of physicochemical properties to help identify biocompatible multifunctional nanoparticles for nanomedicine (Prina-Mello et al. 2012). Moreover, despite some challenges—for example, the possibility that the optical properties of nanoparticles can interfere with fluorescence measurements—HTS methods are also being used in toxicity testing of nanomaterials (Collins et al. 2017). An elegant example was presented by Anguissola et al., who used several organ-specific cell lines for addressing the exposure, accumulation, and clearance of nanoparticles for HCS of previously characterized amine-modified polystyrene nanoparticles. Their platform, which simultaneously measured several cellular parameters in high-throughput format, appeared to match the sensitivity of the assays recommended by the Organization for Economic Cooperation and Development (OECD) guidelines for nanoparticle testing (Anguissola et al. 2014).

In parallel to the rise of nanoparticle use for diagnostics, therapy, and underlying research purposes, recent years have seen an increase in the use of omics technologies for toxicology assessment of nanoparticles (Fröhlich 2017). Thus far, while most studies on assessing the epigenetic impact of nanomaterials have focused on environmental exposure (Stocco et al. 2013), the possible health risks of epigenomic changes triggered by nanomedicine applications merit recognition. Recent findings indicate that both short and long interspersed nuclear elements (SINE [particularly Alu] and LINE, respectively) are targets of epigenetic

modulation in response to nanoparticle exposure (Sooklert et al. 2019) and that the changes in the methylation levels of transposable elements occur after even short-term exposure (Lu et al. 2016). While studies have shown that specific loci in the genome can be affected by methylation changes in response to nanoparticle exposure, the discrepancies in the microarray-based findings of individual studies render any conclusion unsubstantiated. Moreover, because of the limitations of current population-based studies and the lack of genome-wide analysis of epigenetic changes (as opposed to a single type of epigenetic mark reported in the majority of studies) (Smolkova, Dusinska, and Gabelova 2017), there is no evidence yet of the direct effects of nanoparticle exposure on disease risk in humans. Finally, epigenetics marks other than DNA methylation should not be neglected when assessing the effects of nanoparticles (Stoccoro et al. 2013).

Transcriptomics analyses of engineered nanomaterials have yielded an impressive volume of molecular data points for risk assessment in a wide range of organisms (including yeast, worms, fish, and mammals) and in rodent tissues (Rosenblum and Peer 2014, Nayak et al. 2018). In a previous study, a global gene expression analysis of astrocytes, as part of a triple coculture model of the blood–brain barrier, was used to obtain mechanistic insights into silver nanoparticle-induced toxicity. While the number of differentially expressed genes was very low in the treated cultures, GO and KEGG pathway analyses pinpointed several biological processes that could be associated with tight-junction disruption and astrocyte neurotoxicity (Xu et al. 2015). Table 12.1 lists other studies that have used transcriptomic analysis to better understand the mechanisms of toxicity of nanoparticles that have been considered for nanomedicine applications.

Proteomics analysis is also taking its place in toxicological assessment strategies for nanoparticles in medical applications. For investigating the mechanisms of resistance to abraxane, the albumin-bound nanoparticle form of paclitaxel, Zhao et al. conducted a quantitative proteomic analysis of abraxane-sensitive and -resistant A549 cells. Proteins showing differential abundance in response to treatment were analyzed against the KEGG and GO databases, and the results provided a novel inkling that lipid metabolism could be involved in drug resistance only to nanoparticle-coupled paclitaxel (Zhao et al. 2015). Wang et al. used LC-MS/MS-based proteomics analysis to elucidate the molecular mechanisms underlying the cytotoxic effects of ZnO nanoparticles, which are used in diet additives and cosmetics as well as in the diabetes and cancer fields. In addition to demonstrating the downregulation of cytochrome c and related proteins, the authors used GO and KEGG pathway analyses and the STRING database to identify the impacted pathways and association networks of differentially expressed proteins in ZnO nanoparticle-treated murine photoreceptor cells (Wang et al. 2018).

Approaches that use multiple omics platforms can be even more powerful by allowing to confirm and complement each other's findings. A recent study demonstrated good alignment of RNAseq and proteomics analyses indicating mitochondrial dysfunction in undifferentiated human THP-1 cells in response to gold nanoparticles (Gallud et al. 2019). In the following section, we introduce our network scoring approach in the context of toxicological assessment of nanomedicines.

TABLE 12.1

Studies Using Transcriptomics for Nanoparticle Assessment

Study Description	Dataset ID	NP Type	Tissue/Cell	References
Comparative analysis of 3D human bronchial epithelial model exposed to air or CuO nanoparticles reveals asthma-enhanced airway sensitivity to nanoparticles	GSE127773	CuO NPs	MucilAir	Kooter et al. (2019)
Cellular cocultures represent an enhanced <i>in vitro</i> models for nanoparticle risk assessment in multi-walled carbon nanotube exposure setting	GSE129640	Global MWCNT	HMVECs and SAECs	Snyder-Talkington et al. (2019)
Nanotoxicity mechanisms of superparamagnetic iron oxide nanoparticles are described in nematode model	GSE93187, GSE93186	SPION	<i>Caenorhabditis elegans</i>	Gonzalez-Moragas et al. (2017)
Network analysis reveals similar transcriptomic responses to six different carbon nanomaterials <i>in vitro</i> and <i>in vivo</i>	GSE92901, GSE92900, GSE92899	Carbon NMs	THP-1 and mouse lung tissue	Kinaret et al. (2017)
Cytotoxic effects of hydroxylated-graphene quantum dots are presented in human esophageal epithelial cells	GSE96720	Graphene quantum dots	Esophageal epithelial cell line	Li et al. (2018)
Exposure of neurospheres to polyamidoamine (PAMAM) dendrimers affects cell proliferation and migration	GSE65875	PAMAM dendrimer	Neurospheres	Zeng et al. (2016)
Cobalt and cerium dioxide nanoparticles elicit heterogeneous responses in two lung epithelial cell models	GSE45763	CoO- and CeO ₂ -NPs	A549 and BEAS-2B cells	Verstraelen et al. (2014)
Surface charge and chemistry of gold nanoparticles greatly influence the types of affected cellular pathways and the degree of expression change	GSE56432	Gold NPs	HDF cells and PC3 cell line	Grzincic et al. (2015)
Gene-by-gene and gene set analyses demonstrate specific cell response in relation to silica nanoparticles' size and elapsed time after treatment, with the smaller NPs (SM30) having higher impact on inflammatory and apoptotic processes than the bigger ones	GSE53700	Ludox® SM30 and AS30 NPs	A549 cells	Fede et al. (2014)

(Continued)

TABLE 12.1 (Continued)
Studies Using Transcriptomics for Nanoparticle Assessment

Study Description	Dataset ID	NP Type	Tissue/Cell	References
Surface coatings confer almost complete protection against zinc oxide nanoparticle-induced cytotoxicity in human hepatic stellate cells	GSE60159	Coated and uncoated ZnO NPs	hHSCs	Osmond-McLeod et al. (2014)
Amine-terminated dendrimers elicit non-specific toxicity, and the silica nanoconstructs induce gene expression consistent with cell cycle arrest and pro-inflammatory gene induction in human aortic endothelial cells	GSE35142	SiO ₂ , PAMAM dendrimers	HAECs	Moos et al. (2013)
Zebrafish embryos react to PAMAM dendrimers with the activation of innate immune response	GSE41333	PAMAM dendrimers	Zebrafish embryo	Oliveira et al. (2014)
Maternal exposure to carbon black nanoparticle partially suppresses the immune system development of offspring mice	GSE50432	Carbon black NPs	Mouse embryo	Shimizu et al. (2014)
Biological processes regulating cell death, cell growth, and immune system development are affected upon exposure to zinc oxide and titanium dioxide nanoparticles	GSE39330, GSE39316	ZnO and TiO ₂ NPs	HMDM, MDDC, Jurkat	Tuomela et al. (2013)
The toxicity caused by silver nanoparticles is associated with bioavailable silver ions in exposed zebrafish embryos and affects oxidative phosphorylation and protein synthesis pathways	GSE38125	Silver NPs	Zebrafish embryos	van Aerle et al. (2013)
Three human cell types respond to multi-walled carbon nanotubes and titanium dioxide nanobelts with changes in two distinct sets of biological pathways as indication of low or high nanoparticle toxicity	GSE42066, GSE42067, GSE42068	MWCNT, titanium dioxide nanobelts	Caco-2/ HT29-MTX	Tilton et al. (2014)
Exposure to citrate-coated gold NPs caused activation of genes involved in unfolded protein response and endoplasmic reticulum stress, apoptosis, necrosis pathways and resulted in 10% mortality in a nematode model	GSE32521	Gold NPs	<i>C. elegans</i>	Tsyusko et al. (2012)

(Continued)

TABLE 12.1 (Continued)**Studies Using Transcriptomics for Nanoparticle Assessment**

Study Description	Dataset ID	NP Type	Tissue/Cell	References
Superparamagnetic iron oxide NP labeling of bone marrow stromal cells does not alter the differentiation potential of the subset of stem cells within BMSCs	GSE20431	Superparamagnetic iron oxide NPs	BMSCs	Balakumaran et al. (2010)
Gold NPs activate immune-related genes and pathways in human peripheral blood mononuclear cells, but not an immortalized, lineage-restricted cell line	GSE20677	Gold NPs oligonucleotide complexes	PBMCs	Kim et al. (2012)
Exposing macrophages to carbosilane dendrimer or its complex with a random siRNA causes alterations in immune response, proliferation, and transcription regulation	GSE12405	Carbosilane dendrimer: 2G-NN16	Macrophages	Gras et al. (2009)
C60 fullerene particles seem to forgo a severe pulmonary toxicity in contrast to acute inflammation induced by ultrafine nickel oxide particles	GSE13249	C60 fullerene, ultrafine NiO particles	Rat lung	Fujita et al. (2009)
Microarray-based gene expression profiling may aid to identification of potential biomarkers in rat lungs intratracheally instilled with C60 fullerenes	GSE17747	C60 fullerene	Rat lung	Fujita et al. (2010)
This study established a cell line resistant to abraxane, the nanoparticle formulation of anticancer drug paclitaxel through up-regulation of P-glycoprotein	GSE56742	Abraxane, the NP formulation of paclitaxel	A549	Zhao et al. (2015)

Note: The list is limited to studies that were also published in a manuscript.

NP, nanoparticle; NM, nanomaterial; MWCNT, multi-walled carbon nanotube; hHSCs, primary human hepatic stellate cells; HAECs, human aortic endothelial cells; HMDM, human monocyte-derived macrophages; MDCC, monocyte-derived dendritic cells; BMSCs bone marrow stromal cells; PBMCs, peripheral blood mononuclear cells.

12.5 NETWORK IMPACT OF SURFACE-MODIFIED GOLD NANOPARTICLES ON TWO MODEL HUMAN CELL LINES

Recently, we have showcased how cause-and-effect biological network models can be used for meta-analysis of omics data from nanoparticle-treated mouse lungs. Instead of grouping gene expression changes into high-level biological processes, we inferred the activity of individual nodes in the context of biological network, which revealed divergent responses to sanding dust-coupled and free titanium dioxide nanoparticles. This sensitive analysis also identified an unexpected inflammatory signaling response to sanding dust alone (Talikka et al. 2019).

To further illustrate the power of a systems biology approach using CBN model scoring for toxicity evaluation of nanomedicine products, we selected a transcriptomics study of human cell lines exposed to gold nanoparticles with different surface modifications (Grzincic et al. 2015). Gold nanoparticles are attractive vectors for nanomedical applications because of their highly tunable surface functionalities, controlled and wide variety of shapes and sizes, and traceability in biological systems (owing to the physical and chemical properties of gold) (Li and Lane 2019). Because of their unique characteristics, gold nanoparticles are attractive for almost all nanomedical applications, including diagnostics, prevention, therapy, vaccine research, and hygiene (Carabineiro 2017). Different surface coatings of gold nanoparticles can affect cellular biology distinctively. In our selected case study (GSE56432), the authors used four differently functionalized gold nanoparticles with cationic, anionic, and biomimetic lipid-based surface coatings in human dermal fibroblast (HDF) cells and prostate cancer cells (PC3) (Yang, Lohse, and Murphy 2014, Grzincic et al. 2015). HDF cells were chosen to represent unintentional exposure to gold nanoparticles via the skin, and PC3 cells were chosen to represent intentionally targeted cells for imaging or therapy. The cells were exposed to media containing 20nm gold nanoparticles surface-coated with citrate (CIT), alkanethiol/lipid (ATL), poly(allylamine hydrochloride) (PAH), or poly(allylamine hydrochloride)/lipid (PAHL). The samples were collected in triplicate, and transcript abundances were analyzed by using the Agilent gene expression platform. The authors compared the number of genes that showed differential expression in response to the nanoparticle exposure in HDF and PC3 cells and used DAVID to identify the impacted biological processes. The genes associated with NF κ B (nuclear factor 'kappa-light-chain-enhancer' of activated B cells) appeared to be largely involved in gold nanoparticle response.

Here, we present the results of scoring of relevant models (e.g., cell fate, proliferation, and cell stress) that were impacted in at least one of the treatments in the dataset. The network heatmap provides an overview of how the different treatments affected the cells relative to treatment with the vehicle (Figure 12.4a). Several of the tested network models were impacted in cell cultures treated with gold nanoparticles, and all tested formulations elicited significant perturbation of the senescence network model, with the highest impact by PAHL in both HDF and PC3 cells. In HDFs, the effects of ATL and CIT were generally lower than those of PAH; however, in PC3 cells, ATL perturbed more networks than PAH.

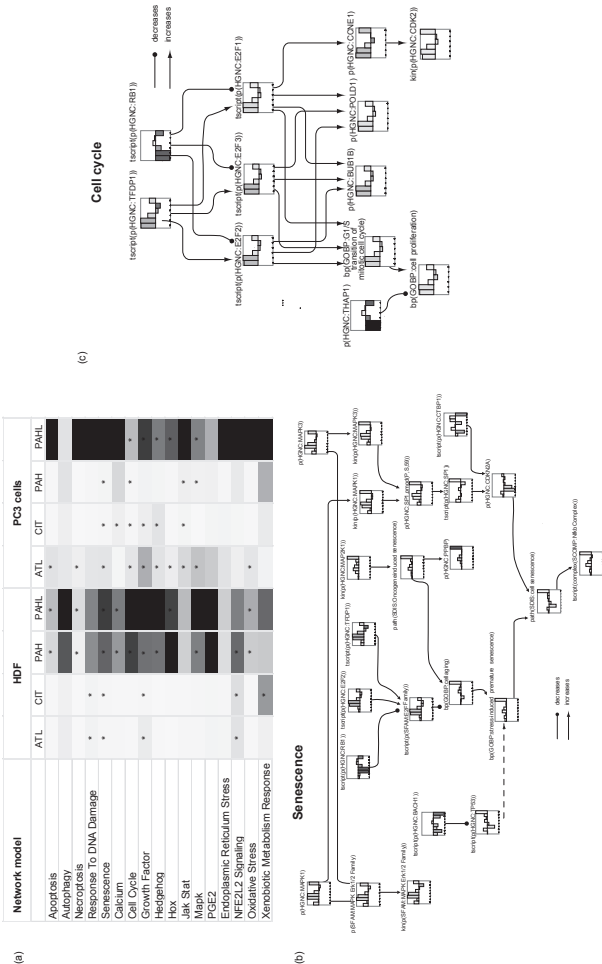


FIGURE 12.4 Network impact of surface-modified gold nanoparticles in two model human cell lines. (a) Network perturbation amplitude (NPA) in response to gold nanoparticle treatment in HDFs and PC3 cells. The darker the shading, the greater the impact on the network model. *O and K statistic p values are less than 0.05, and the NPA is significant with respect to the experimental variation. (b) Subgraphs of the senescence network model, depicting the common leading nodes in response to gold nanoparticle treatment in HDFs and PC3 cells. The bar graphs indicate the backbone NPA values, with directionalities of inferred regulation for each node (Martin et al. 2019a). The chart can be read as follows: The left-most column represents the contrast of (1) ATL/HDF, (2) CIT/HDF, (3) PAH/HDF, (4) PAHL/HDF, (5) ATL/PC3, (6) CIT/PC3, (7) PAH/PC3, and (8) PAHL/PC3. (c) Subgraphs of the cell cycle network model depicting the common leading nodes in response to gold nanoparticle treatment in HDFs and PC3 cells. The bar graphs indicate the backbone NPA values, with directionalities of inferred regulation for each node (Martin et al. 2019a). The chart can be read as follows: The left-most column represents the contrast of (1) PAH/HDF, (2) PAHL/HDF, (3) ATL/PC3, (4) CIT/PC3, (5) PAH/PC3, and (6) PAHL/PC3. In (b) and (c), orange/red and blue bars indicate inferred upregulation and downregulation, respectively. The dashed edge indicates an indirect path in the network model (entities among the leading nodes are not shown). The entire senescence network model can be downloaded from [causalbionet.com](https://bel.bio/), and the BEL terminology can be found in <https://bel.bio/>.

For a more detailed investigation, we selected the senescence network model, which was impacted by all treatments in both cell lines. The results of the leading node analysis pinpoint the regions of the network model that are the major contributors for the observed effect (Martin et al. 2019b). We have extracted the common leading nodes from each comparison and created a subgraph. Figure 12.4 shows the magnitude and directionality of the effect that each treatment was inferred to have had on the different molecular entities in the senescence model. The strong inferred downregulation of the transcription factor BACH1 (BTB and CNC homology 1) by PAHL was reflected in the upregulation of TP53 in PC3 cells. In general, the treatment with PAHL appeared to induce senescence/cell aging in PC3 cells as well as strong upregulation of the pro-platelet basic protein (PPBP). Retinoblastoma protein (RB1) was inferred to be downregulated by both PAH and PAHL in HDF cells, resulting in upregulation of the E2F family, suggesting a more proliferative rather than a senescence-inducing effect. In addition, MAPK family factors were inferred to be upregulated in both cell lines by all treatments, with the exception of PAH treatment in PC3 cells (Figure 12.4b). The inferred proliferative effect was revealed by investigation of the cell cycle network model, which shares several nodes with the senescence model. The cell cycle network model was not significantly impacted by ATL or CIT in HDF cells. As shown in Figure 12.4c, with the exception of CIT and PAH in PC3 cells, transcription factor Dp-1 (TFDP1) and the E2F factors were inferred to be upregulated, indicating increased cell proliferation.

In summary, NPA scoring and leading node analysis allowed us to compare the impact of different nanomedicine formulations on biological processes and also dissect the convergent and divergent effects they have on the molecular players in various signaling pathways in different cell types. While the original study identified NF κ B as a molecular target, we also observed indication of a proliferative signal in response to gold nanoparticle treatment. Hence, in contrast to simple pathway analysis, the network approach helps interpret molecular changes in the context of known biological processes.

12.6 CONCLUSIONS AND OUTLOOK

One of the core challenges that the US Food and Drug Administration (FDA) faces today is the identification of novel risks, and the various unique properties of nanoparticles that make them so attractive for medical applications also pose new challenges for risk assessment. The process of characterizing nanoscale materials and quantifying their impact necessitates new toxicological assessment and testing tools (Paradise 2019, Resnik 2019)—this is a space to which systems biology-based methods can and should contribute. First, measurement of thousands of molecular species gives much more insights into toxicity mechanisms than any apical endpoint. This approach conforms to the 21st-century toxicology paradigm, which shifts toxicology research from animal tests to toxicity pathway assessment in cells and tissues (Langley et al. 2015). Second, because the effects of nanoparticle exposure can be subtle and take long to manifest, application of computational models for predicting the toxicological risks of nanoparticles opens new avenues

in nanomedicine safety research. Instead of testing every single nanoparticle individually, bioinformatics and computational approaches allow us to build comprehensive toolsets for predicting the harmful effects of nanoparticles on humans and the environment (Furxhi et al. 2019, Pikula et al. 2019, Stueckle and Roberts 2019).

The sensitivity of people to any toxicant is variable; this variation is not only due to sex, ethnicity, body weight, or age but can also stem from interindividual differences in molecular profiles that impact their organ functions. While genotyping can give certain insights, profiling of gene products (e.g., mRNA and proteins) and cellular metabolites provides a more comprehensive view of an individual's overall (baseline) sensitivity to foreign substances. In fact, personalized and precision toxicology deal with such biological complexity that none other than systems approaches are equipped to handle it. The increasing availability of big data also allows the development and training of computational models that can help in dynamic subject classification on the basis of baseline characteristics that might define individual sensitivity (Ochoa and Weil 2019).

Finally, out of ethical considerations regarding *in vivo* studies—and given the current grim environmental situation, which compels everyone to decrease the amount of waste in our daily activities—we need to take a look at the data from all studies published to date. Unless analyzed and interpreted in an exhaustive manner, existing data become superfluous. So, why not take advantage of the existing data and use them to implement thorough systems toxicology evaluations based on literature-curated CBN models, a platform that provides unprecedented information for practical toxicological assessment?

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13 Measurement of Oxygen Consumption Rate Based on Fluorescence Intensity and Lifetime as a Strategy to Assess Nanotoxicity

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13.1 INTRODUCTION

Nanotechnology is an emergent field in biomedicine, especially for advanced drug delivery and medical diagnostics (McNamara and Tofail 2017). Because of their size, nanoparticles gain entry across biological barriers and penetrate deeply into tissues (Shang, Nienhaus, and Nienhaus 2014). Their high surface-to-volume ratio also enables nanoparticles to be very reactive/catalytic, cross plasma membranes, and interact with cellular organelles (Unfried et al., 2007). Nanomaterials with diverse physical and chemical properties are implicated in a variety of adverse reactions, including (i) onset of oxidative stress, (ii) DNA damage, (iii) altered cell signaling, (iv) altered cell motility, (v) apoptosis, and (vi) carcinogenesis (Hillegass et al. 2010). The drug-loaded nanoparticles are likely to show added adverse reactions by altering the intracellular bioavailability of drugs (i.e., active pharmaceutical ingredient; API) themselves (Rodrigues de Azevedo et al. 2017). Thus, notwithstanding their advantages for drug delivery and diagnostics, nanoparticles have the potential to induce a wide range of adverse effects (De Jong and Borm 2008). The resulting nanotoxicity constitutes a significant impediment for further extension of their novel properties in biomedicine. In addition to particles intended for therapeutics, nanotoxicity can also manifest in response to nanoparticles frequently ingested from the environment via inhalation and direct skin contact (Sajid et al. 2015). The goal of this chapter is to review altered O₂ consumption rate (OCR) as a metabolic measure of nanotoxicity in cells/tissues. The techniques described here should be particularly useful in preclinical studies.

13.2 ASSAYS FOR NANOTOXICITY

During the development of nanoparticles for biomedical applications, nanotoxicity is addressed beginning with preclinical studies. Hence, assays for nanotoxicity necessarily have to be at the level of high-throughput screening (HTS) so that a large variety of candidate nanoparticles can be investigated rapidly. To facilitate an HTS approach, cell culture models are most attractive for testing the toxicological effects of nanoparticles. They are not only cost-effective but also obviate animal testing. Accordingly, commonly sought after nanotoxicity assays include determining cell death, the release of cellular enzymes (e.g., LDH), or loss of cell membrane integrity (Hynes et al. 2006). Cell membrane damage and cell death are late-stage indicators of cytotoxicity. However, changes in the cellular metabolism often precipitate before disrupting the cell membrane (Hillegass et al. 2010). Therefore, an assay of parameters affected by cell metabolism would be expeditious for analyzing cytotoxicity. The extracellular acidification (ECA) and OCR are two parameters that have been routinely assayed with high sensitivity using techniques that are suitable for HTS (Hynes, Swiss, and Will 2012). ECA is

a consequence of lactate accumulation, which occurs when metabolism switches to glycolysis. Thus, ECA is enhanced when cellular toxicity results in mitochondrial damage. On the other hand, an analysis of OCR reflects direct damage to mitochondria or cell death (Prill et al. 2016).

13.3 TECHNIQUES FOR MEASUREMENT OF OCR

O₂ metabolism is essential in all cells for deriving energy from the complete oxidation of glucose (Figure 13.1a). In particular, O₂ participates in oxidative phosphorylation (OxPhos) that takes place in mitochondria (Figure 13.1b). It acts as a terminal electron acceptor in the electron transport chain (ETC), in which oxidation of NADH is coupled to generation of ATP. NADH, as a reducing equivalent, is produced in the tricarboxylic acid (TCA) cycle, which also takes place in the mitochondria. Thus, any impact on OxPhos-mediated OCR results in intra- and extracellular changes in pO₂. Therefore, altered OCR is a sensitive index of viability and metabolic status of cells/tissues. In other words, intra- or extracellular pO₂ measurements are suitable biomarkers to establish mitochondrial health (Mik, Balestra, and Harms 2020), and hence nanotoxicity. Also, an altered pO₂ can have many biological consequences. For example, a decrease in pO₂ can induce mobilization of transcription factor HIF-1 α (Masson and Ratcliffe 2014). Specifically, reduced pO₂ stabilizes HIF-1 α which results in proliferation, migration, and morphogenesis of vascular endothelial cells leading to angiogenesis (Cash, Pan, and Simon 2007) (Figure 13.2). Taken together, in the absence of obstruction to local oxygenation, any disturbance to pO₂ in a biological micro-environment is indicative of mitochondrial dysfunction, altered energy metabolism, or cell death. While an assay of altered HIF-1 α is imperative to determine changes in local oxygenation, dynamic measurements of pO₂ in tissues/cells, and hence OCR, is a robust and real-time approach to evaluate nanotoxicity.

13.4 O₂ SENSING TECHNIQUES

OCR can be determined by measuring changes in pO₂ in the immediate micro-environment of the target cells over a finite period held in a closed volume (Plitzko and Loesgen 2018). The sensitivity of OCR measurements can be enhanced by reducing the volume of the medium bathing the cells. The time course of pO₂, necessary for the estimation of OCR, can be measured by a number of techniques: (i) electrochemical methods (i.e., polarography), (ii) electron paramagnetic resonance (EPR), (iii) fluorescence of NADH (qualitative only), and (iv) fluorescence of pO₂-sensitive dyes. The method of polarography uses electrodes and hence would be invasive for local pO₂ assessment in biological systems. Moreover, the electrochemical methods consume O₂ (Swartz et al. 2003; Roussakis et al. 2015) and are questionable for HTS. EPR oximetry is a minimally invasive method and has been employed to examine pO₂ in the intact beating heart (Zweier, Thompson-Gorman, and Kuppusamy 1991; Kuppusamy et al. 1994), brain (Swartz et al. 2003), gastrointestinal tract (He et al. 2004), skeletal muscle (Ilangoan et al. 2002), liver

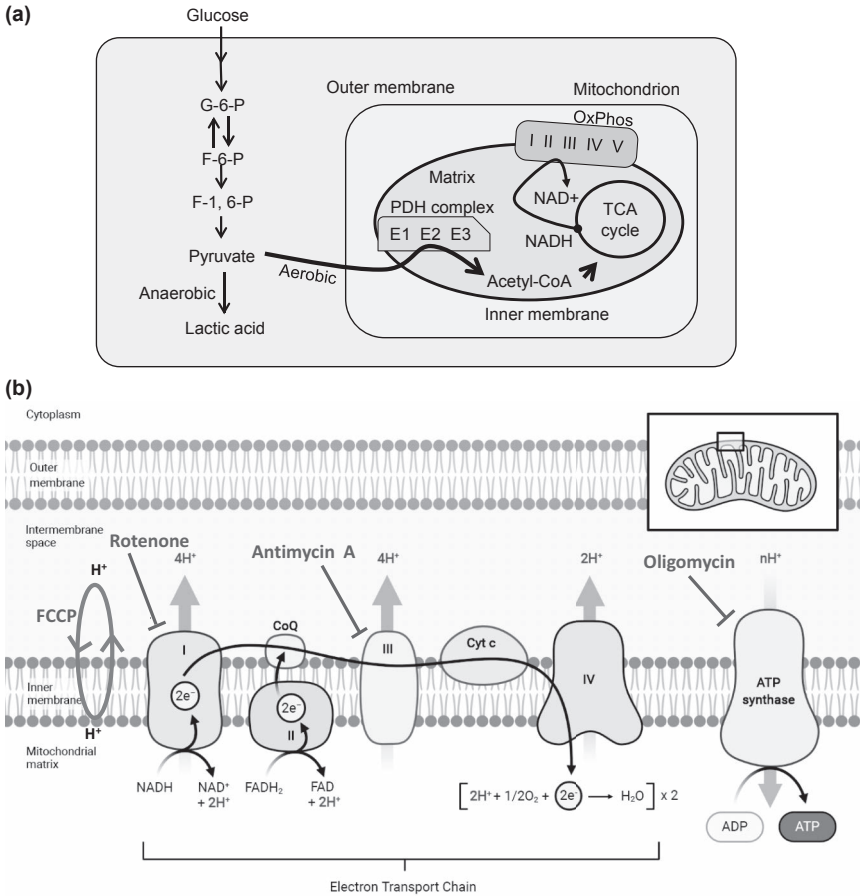


FIGURE 13.1 O₂ metabolism. O₂ is consumed mainly by oxidative phosphorylation (Ox-Phos) in the mitochondria. The metabolic pathway of glycolysis converts glucose to pyruvate, which then generates acetyl-CoA. The oxidation of acetyl CoA by Ox-Phos results in energy production. (a) In Ox-Phos, electrons are transferred on the electron transport chain (ETC) to create proton gradients across the inner mitochondrial membrane, which is utilized in ATP production by F1/F0-ATP synthase. (b) ETC components and its known inhibitors—oligomycin, FCCP, antimycin A, and rotenone. All these agents affect OCR.

(Madhani et al. 2002), kidneys (James et al. 1997; James, Thomas, and Jackson 2004), and skin (He et al. 2004). However, the main disadvantage of EPR is its lack of spatial resolution and is not developed for HTS.

Although NADH fluorescence cannot be directly correlated to extracellular pO₂, its non-invasive measurements can provide an index of the metabolic status of mitochondria in terms of energy production and intracellular pO₂. These principles have been demonstrated in a large number of studies at the level of single cells, *ex vivo* tissues, and intact organs (Ince, Coremans, and Bruining 1992;

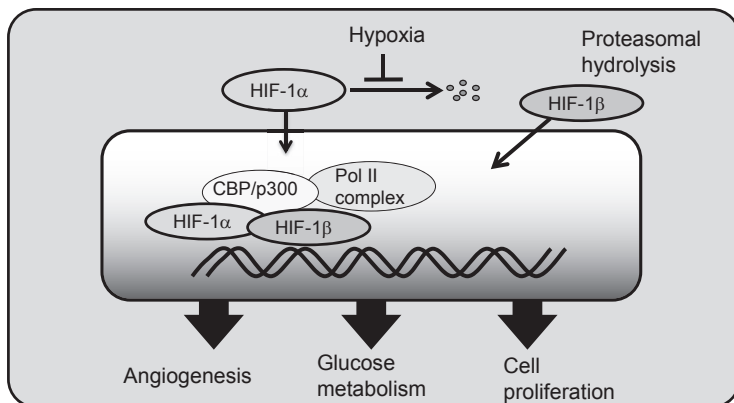


FIGURE 13.2 Biochemical O_2 sensing: low pO_2 stabilizes HIF-1 α , which forms a heterodimer with HIF-1 β . In normoxic conditions, HIF-1 α is ubiquitinated and thus undergoes proteasomal hydrolysis. The heterodimer HIF-1 elicits activation of gene expression responsible for angiogenesis to counter hypoxia. In addition, glucose metabolism and cell proliferation are induced. Detection of elevated HIF-1 α can be a strategy to assess nanotoxicity, which affects oxygenation in a tissue of interest.

Ince et al. 1992; Mayevsky and Rogatsky 2007). A typical example of application to intact tissue *ex vivo* is exemplified by the study of transcorneal redox assessment in rabbit cornea as shown in Figure 13.3a. While the technique is useful to validate the other methods of nanotoxicity, it is not suitable for HTS assays. UV excitation at ~ 360 nm, the peak wavelength for NADH excitation (Figure 13.3b), is also a technical impediment since UV penetration deeper into tissues would be limited (Sennhenn et al. 1993). Prolonged UV excitation, moreover, is toxic to cells/tissues. Some of these problems can be circumvented partly by multi-photon techniques. However, the associated costs of lasers limit their use for routine preclinical assays. Finally, the emitted NADH fluorescence is weak, which not only reduces the sensitivity but also necessitates enhanced detection techniques. To overcome these disadvantages, other optical methods based on exogenous pO_2 -sensitive dyes have been advanced (Quaranta, Borisov, and Klimant 2012).

13.5 O_2 -SENSITIVE FLUORESCENT DYES

Several luminescent O_2 -sensitive dyes have been reported for biological pO_2 measurements (Quaranta, Borisov, and Klimant 2012). These can be classified broadly into metalloporphyrins (e.g., Pt(II) and Pd(II)-porphyrins), Ru(II) complexes, and Ir(III) complexes. The porphyrins emit phosphorescence with long lifetimes (10–1,000 μ s) in response to green excitation (O'Donovan et al. 2005). Ru(II) complexes show fluorescence with a long Stokes shift and short lifetimes (0.1–6 μ s) in response to blue excitation (Quaranta, Borisov, and Klimant 2012). In contrast, Ir(III) complexes show intermediate lifetimes (1–15 μ s) (Zhang et al. 2018). Although there are differences in the spectral characteristics, all dyes undergo

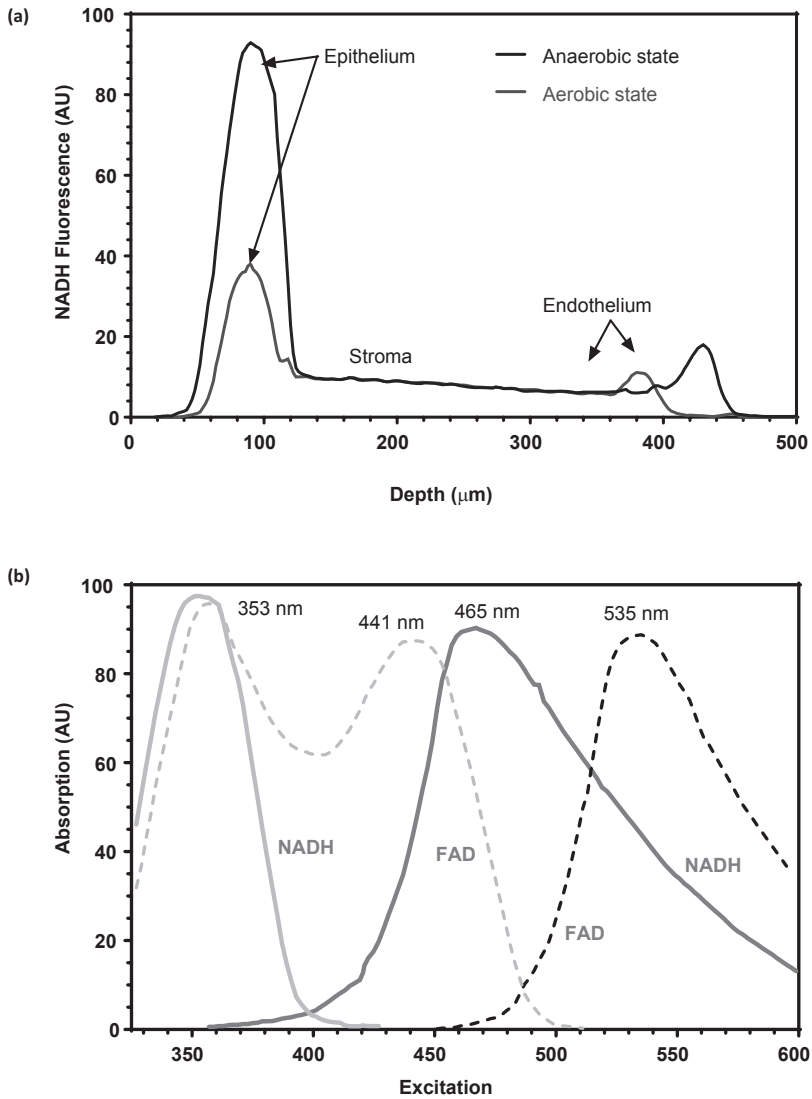


FIGURE 13.3 NADH fluorescence in response to hypoxia: NADH is fluorescent, while its oxidized counterpart NAD^+ is not. Since the relative magnitude of NADH to NAD^+ is regulated by Ox-Phos, the magnitude of NADH fluorescence is affected by mitochondrial activity. (a) Effect of hypoxia on NADH fluorescence in tissues. The transcorneal profile across rabbit cornea *ex vivo* (redrawn from Masters et al. (1984a, 1984b)) is shown to highlight the impact of hypoxic metabolism on NADH fluorescence. (b) Excitation and emission spectra of the redox nucleotides. NADH has fluorescence excitation and emission maxima at 353 and 465 nm, respectively. The fluorophore flavin adenine dinucleotide (FAD) is also fluorescent with excitation and emission maxima at 441 and 535 nm, respectively (Ince, Coremans, and Bruining 1992; Ince et al. 1992).

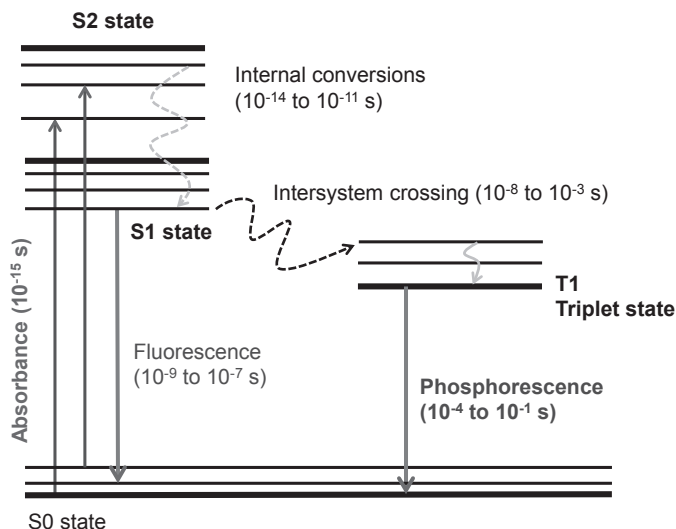


FIGURE 13.4 Jablonski diagram. This diagram illustrates the energy levels of fluorescent and phosphorescent molecules during excitation and emission. Horizontal bars represent energy levels of the different excitation states of the molecule. S_0 = singlet ground state; S_1 = first singlet excited state; S_2 = second singlet excited state; T_1 = triplet excited state. Upon absorption of excitation, electrons are raised to a higher energy excited state. The excited molecules emit photons and fall back into lower energy states through fluorescence or phosphorescence. The latter requires the forbidden intersystem crossing, which is shown by dotted lines. Since part of the energy in the excited state is lost by non-radiative processes, the fluorescence and phosphorescence are red-shifted. Porphyrins show phosphorescence, while Ru(II) and Ir(III) complexes yield fluorescence following excitation. The wave arrows indicate transitions that involve non-radiative relaxation processes.

dynamic quenching by molecular O_2 (Figure 13.4). Thus, quenching increases with an increase in pO_2 and *vice versa* (Quaranta, Borisov, and Klimant 2012). Specifically, the relationship between pO_2 and the altered emission intensity follows the Stern–Volmer relationship (Eq. 13.1):

$$\frac{F_0}{F} = 1 + K_{SV}pO_2 \quad (13.1)$$

where F_0 is the observed fluorescence when $pO_2 = 0$ and K_{SV} is the Stern–Volmer constant.

The ideal requirements of an O_2 probe are high photostability, simple, and efficient means of delivery into the cell/tissue, minimal cytotoxicity, and optimal photophysical properties. Moreover, pO_2 changes can be made with O_2 -sensitive probes in the culture medium or incorporated into cells/tissues. While using the latter approach, it is imperative to ensure that the O_2 -sensitive dye is not

toxic to the target cells/tissues. Dobrucki (2001) reported that tris(2,2'-bipyridyl) ruthenium(II) chloride hexahydrate ($\text{Ru}(\text{bipy})_3^{2+}$) and tris(1,10-phenanthroline) ruthenium(II) chloride hydrate ($\text{Ru}(\text{phen})_3^{2+}$) do not cause measurable photo-damage to plasma membranes at a concentration of 0.2 mM. This was attributed to the fact that the dye did not pass through intact biological membranes. Tables 13.1–13.3 list representative pO_2 -sensitive dyes that have been tested in various cells and tissues.

TABLE 13.1

Representative O_2 -Sensitive Porphyrin Derivatives (Lifetime in the Range of ms when $\text{pO}_2 = 0$ mm Hg)

Dye	λ (nm)		Formulation	Measurement Site	References
	Ex	Em			
Pt(II) meso-tetrakis(pentafluorophenyl) porphine (PtTFPP)	532	600–700	Dispersed in poly perfluoroether (PFPE)	Intracellular	Zhao et al. (2018)
Pt-tetraphenylporphyrin (PtTPP)	514	648	Stock solution: 1 mM Pt-TPP and 0.1 M tetrabutylammonium perchlorate in acetonitrile	Tissue	Holmes-Smith et al. (1999)
Pd-meso-tetra-(carboxyphenyl)-prophyrin (PTP) (Oxyphor R2)	415, 524	690	Dissolved in saline containing 1.5% BSA (bovine serum albumin)	Tissue	Dunphy, Vinogradov, and Wilson (2002); Papkovsky and Zhdanov (2016)
Pd-meso-tetra-(4-carboxyphenyl)tetra benzoporphyrin dendrimer (Oxyphor G2)	440, 632	790	Dissolved in saline containing 1.5% BSA	Tissue	Dunphy, Vinogradov, and Wilson (2002)
Palladium-(II) coproporphyrin ketone (PdCPK)	405	796	In PBS (100 μM stock)	Intracellular	O'Riordan et al. (2007a, 2007b)
Pt tetrakis(4-carboxyphenyl) porphyrin (PtTCPP)	405/515	435/525	Dissolved in DMSO	Intracellular	Kurokawa et al. (2015)
Platinum-(II) coproporphyrin ketone (PtCPK)	399	767	In PBS (100 μM stock)	Intracellular	O'Riordan et al. (2007a, 2007b)
Platinum-(II) octaethylporphyrin ketone (PtOEPK)	395, 586	760	Polystyrene	Intracellular	O'Donovan et al. (2005)

(Continued)

TABLE 13.1 (Continued)**Representative O₂-Sensitive Porphyrin Derivatives (Lifetime in the Range of ms when pO₂ = 0 mm Hg)**

Dye	λ (nm)		Formulation	Measurement	
	Ex	Em		Site	References
Pd-coproporphyrin (PdCP)	393	670	Solution	Tissue	Papkovsky and O'Riordan (2005); Papkovsky and Dmitriev (2013); Papkovsky and Zhdanov (2016)
Platinum(II) octaethylporphyrin (PtOEP)	382, 540	650	Polystyrene	Intracellular	O'Donovan et al. (2005)
Pt-coproporphyrin (PtCP)	380	646	In PBS (100 μ M stock)	Intracellular	O'Riordan et al. (2007a, 2007b)
Pt(II) tetrakis(pentafluorophenyl) porphyrin (PtTFPP)	340	642	Encapsulated in cationic Eudragit nanoparticles RL-100 (particle size ~30 nm)	Intracellular	Fercher et al. (2011)

TABLE 13.2**Representative O₂-Sensitive Ru(II) Derivatives (Lifetime in Solution ~0.1–10 μ s)**

Dye	λ (nm)		Formulation	Measurement	
	Ex	Em		Site	References
Tris(2,2'-bipyridyl) ruthenium(II) chloride hexahydrate [Ru(bipy) ₃] ²⁺	485	610 $\pm$ 10	In deionized water at a concentration of 13.34 mM	Pericellular	Hosny, Lee, and Knight (2012)
Ruthenium dibipyridine 4-(1"-pyrenyl)-2,2'-bipyridine chloride [(bpy) ₂ Ru(bpy-pyr)]Cl ₂	456	632	PBS	Intracellular	Ji et al. (2002)

(Continued)

TABLE 13.2 (Continued)
Representative O₂-Sensitive Ru(II) Derivatives (Lifetime in Solution ~0.1–10 μs)

Dye	λ(nm)		Formulation	Measurement	References
	Ex	Em		Site	
Ru(II)-tris(4,7-diphenyl-1,10-phenanthroline) chloride ([Ru(dpp) ₃] ²⁺)	450, 543	605–650	Doped in organically modified silica nanoparticles (70 nm in diameter)	Intracellular	Korzeniowska et al. (2013, 2015)
Tris(1,10-phenanthroline) ruthenium(II) chloride [Ru(phen) ₃]	450	604	Aqueous solution	Intracellular	Asiedu et al. (2001)
Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride complex (Ru(dpp) ₃ Cl ₂)	450, 498	608	Encapsulated in plasticized polymethyl methacrylate (PMMA) doped with Ag nanoparticles	Tissue	Jiang et al. (2017)
Ruthenium tris(2,2′-dipyridyl) dichloride hexahydrate (RTDP)	450	620	PBS (0.13 mM)	Intracellular	Sud and Mycek (2009)
Ruthenium-complex Kr341	449	638	Dissolved in DMSO	Intracellular	Jahn, Buschmann, and Hille (2015)

TABLE 13.3
Representative O₂-Sensitive Iridium Derivatives (Lifetime in Solution ~0.1–20 μs)

Dye	λ (nm)		Formulation	Measurement	References
	Ex	Em		Site	
Iridium(III) complex BTP, (btp)2Ir(acac) (btp = benzothienylpyridine, acac = acetylacetone)	485	615	–	Intracellular	Yoshihara et al. (2015, 2017)
Iridium(III) complex with 3-(5-chlorobenzooxazol-2-yl)-7-(diethylamino)-coumarin Ir(C ₈) ₂ (acac)	472	563	Dissolved in PS and chloroform	Intracellular	Borisov and Klimant (2007)

(Continued)

TABLE 13.3 (Continued)

Representative O₂-Sensitive Iridium Derivatives (Lifetime in Solution ~0.1–20 μ s)

Dye	λ (nm)		Formulation	Measurement Site	References
	Ex	Em			
Iridium(III) complex with 3-(5-chlorobenzooxazol-2-yl)-7-(diethylamino)-coumarin Ir(C _O) ₂ (acac)	467	552	Dissolved in PS and chloroform	Intracellular	Borisov and Klimant (2007)
Iridium(III) complex with 3-(5-chlorobenzooxazol-2-yl)-7-(diethylamino)-coumarin Ir(C _N) ₂ (acac)	450	545	Dissolved in PS and chloroform	Intracellular	Borisov and Klimant (2007)
Tris(2-phenylpyridine anion) iridium(III) Ir(ppy) ₃ ²⁺	376	512	Immobilized in a fluoropolymer (poly(styrene-co-TFEM)) film	Intracellular	Amao, Ishikawa, and Okura (2001); Fernandez-Sanchez et al. (2007)

13.6 SENSING pO₂ BASED ON FLUORESCENCE INTENSITY

As noted by Eq. (13.1), a direct approach to measure pO₂ using a pO₂-sensitive dye is to determine its emission intensity. This approach is suitable when the dye can be uniformly distributed at the site of measurement as in cell culture plates. When O₂ probes are encapsulated in nanoparticles, the particle matrix serves as a physical barrier between the probe and the biological milieu. They reduce the potential toxicity of the probes or their intermediates, and thereby prevent unwanted interference or degradation with intracellular organelles/enzymes. Xu et al. (2001) prepared sol–gel nanoparticles, referred to as probes encapsulated by biologically localized embedding (PEBBLES), for measurements of intracellular pO₂ in rat C6 glioma cells. PEBBLES incorporated O₂-sensitive tris (4,7'-diphenyl-1,10'-phenanthroline)ruthenium(II) [Ru-(dpp)₃]²⁺ and Oregon Green 488-dextran (an oxygen O₂-insensitive dye; as a reference for ratiometric intensity measurements). They showed a high dynamic range as well as stability against leaching and photobleaching. The dyes held in the matrix of the particles did not interfere with proteins in cells, indicating reduced or no toxicity of the dyes so that nanotoxicity can be assayed effectively. Similar to PEBBLES, Wang et al. (2013) have encapsulated Ru complex into polystyrene nanoparticles and have successfully demonstrated imaging of O₂ distribution in cells and tissues. Fercher et al. (2011) demonstrated a nanoparticle formulation of Pt(II)-tetrakis(pentafluorophenyl) porphine (PtPFPP) in a cationic polymer. They recorded metabolic responses of mouse embryonic fibroblast cells under ambient and hypoxic microenvironment using a time-resolved fluorescence microplate reader. Ji et al. (2002) describe the application of ruthenium dibipyridine 4-(1''-pyrenyl)-2,2'-bipyridine chloride

[Ru(bpy-pyr)(bpy)₂] for intracellular pO₂ measurements in J774 murine macrophages. The dye showed strong visible absorption, efficient fluorescence, long excited-state lifetime, large Stokes shift, and high photo and chemical stability.

Apart from the above approaches for intensity measurements (Table 13.4), integrated platforms also exist to measure pO₂ in the HTS scale as microplate readers or dedicated devices. The latter is exemplified by XF analyzer (Agilent, Inc.), which is particularly developed to detect ECA and OCR. It is used with disposable cartridges that contain fluorescent probes for simultaneous measurements of O₂ and pH in a small sealed volume over the cells (van der Windt, Chang, and Pearce 2016). The readouts are in the forms of pO₂ and proton flux, which can be converted into OCR and ECA rate. These directly quantify OxPhos and glycolysis, respectively (de Moura and Van Houten 2014; Lay et al. 2016).

13.7 SENSING pO₂ BASED ON FLUORESCENCE LIFETIME

Since the fluorescence intensity of a fluorophore is affected by its concentration, it is imperative to measure their fluorescence lifetime (Figure 13.5) to assess pO₂ when they cannot be distributed uniformly in the measurement domain of interest (Zhong, Urayama, and Mycek 2003). In the parlance of fluorescence lifetime, Eq. (13.1) can be rewritten as follows:

$$\frac{\tau_0}{\tau} = 1 + K_{sv}pO_2 \quad (13.2)$$

where τ_0 is the observed lifetime when pO₂ = 0 and K_{sv} is the Stern–Volmer constant. Unlike the measurement of fluorescence intensity, which is routine and straightforward, the lifetime measurement is relatively complex, especially in tissues or *in vivo*. In general, the fluorescence lifetime is measured by two distinct approaches, viz. the time-domain (TD) and frequency-domain (FD) methods. In the TD approach, the response to a pulse excitation is measured, while in the FD, the response to modulated excitation is used to determine lifetime.

13.8 TIME DOMAIN (TD) APPROACH

The TD approach is simple, especially when testing the fluorophores of long half-lives. In the TD approach, fluorescence decay in response to pulsed light can be recorded using a multi-channel scaler (Sushma et al. 2019) or by time-correlated single-photon counting (TCSPC) technique (Figures 13.6 and 13.7) (Becker 2007). Direct analog recording would be prohibitive for fast fluorescence decays. Once the fluorescence response to pulse input is obtained, single or double exponential decay models are fitted by non-linear least squares or by less-accurate but fast algorithms (e.g., RLD technique (Chan et al. 2001)). In Figures 13.6 and 13.7, we illustrate our approach for the measurement of depth-resolved fluorescence lifetime using a custom-built confocal scanning microfluorometer (Maurice and

TABLE 13.4
Intensity Based Measurements of Lifetime of pO₂-Sensitive Dyes

Tissue/Cells	pO ₂ -Sensitive Dye	Formulation	Technique	Observations	References
Mouse embryonic fibroblast cells	Pr(II)-tetakis(pentafluorophenyl) porphyrin [PtPFPPI] (Frontier Scientific, UT, USA)	Encapsulated in cationic nanoparticles of Eudragit (RL-100)	Fluorescence intensity imaging λ_{ex} = 340 nm λ_{em} = 642 nm	Probe is self-loading; showed high brightness and photostability Assayed mitochondrial bioenergetics	Fercher et al. (2011)
Rat C6 glioma cells	Platinum(II) octaethylporphine [PtOEP] (Frontier Scientific, UT, USA)	Dyes encapsulated in inert nanoparticle (PEBBLE ^a sensors)	Fluorescence intensity imaging PtOEP, λ_{ex} = 540 nm λ_{em} = 644 nm	Fluorescent ratio increased by 500% in cells when pO ₂ was reduced by 90% Enabled simultaneous pO ₂ and glucose assay	Koo et al. (2004)
Breast adenocarcinoma cell line MCF-7 (human)	Platinum-(II) octaethylporphine ketone [PtOEPK] (custom synthesis)	Ru(dpp) converted to [Ru(dpp) ₃] TMSPS ₂ ^b and solubilized in polystyrene, subsequently formulated into nanoparticles [Ru-PSNP]	Fluorescence intensity imaging PtOEPK, λ_{ex} = 568 nm λ_{em} = 750 nm	Ru-PSNP enabled imaging of intracellular pO ₂ distribution	Wang et al. (2013)
Chlorella vulgaris	Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride complex (Ru(dpp) ₃ Cl ₂) (J&K Chemical Company, Shanghai, China)	Encapsulated in plasticized polymethyl methacrylate (PMMA) doped with Ag nanoparticles	Fluorescence intensity imaging 2-P excitation (laser: 830 nm) λ_{ex} = 488 nm λ_{em} = 650 nm	AgNPs showed good stability, response time, and photostability. Sensor film can be adopted for aqueous conditions	Jiang et al. (2017)

(Continued)

TABLE 13.4 (Continued)
Intensity Based Measurements of Lifetime of pO₂-Sensitive Dyes

Tissue/Cells	pO ₂ -Sensitive Dye	Formulation	Technique	Observations	References
HeLa cells	[Ru(bpy) ₃] ²⁺ metallopolymer (Ru-Poly) (custom synthesis)	O ₂ nanoprobe of O ₂ -sensitive metallopolymer (Ru-Poly) and O ₂ -insensitive metallopolymer (Tb-Poly)	Confocal laser scanning microscopy (ratiometric) λ_{ex} = 488 nm λ_{em} = 585–625 nm	Distribution of intracellular oxygen within 3-D multi-cellular tumor spheroids imaged	Zhao, Zhou, and Peng (2019)
J774 murine macrophages	Ru ²⁺ dibipyridine 4-(1''-pyrenyl)-2,2'-bipyridine chloride [Ru(bpy-pyr)(bpy) ₂] (Aldrich Chemical Company)	In PBS buffer at pH 7.4	2-p excitation at 720nm Fluorescence intensity imaging λ_{ex} = 460nm λ_{em} = 632 nm	Fluorescence intensity increased four-fold when oxygenated solution was replaced with a nitrogenated one O ₂ sensitivity of the dye is maintained for 5 hours after loading	Ji et al. (2002)
J774 Murine macrophages	Tris(1,10-phenanthroline) ruthenium(II) chloride [Ru(phen) ₃] (Aldrich Chemical Company)	1 mL of dye incubated for 15 min such that the dye concentration is 1,024 M in the solution. The cells were then washed with PBS buffer	FLIM λ_{ex} = 450nm λ_{em} = 604 nm	Fluorescence intensity decreased by 30% when exposed to external hypoxia Intensity recovered to 90% of its original value once normal conditions were restored	Asiedu et al. (2001)

^a Photonic explorer for biomedical use with biologically localized embedding.

^b TMSPS2 = di(trimethylsilyl)propanesulfonate) salt.

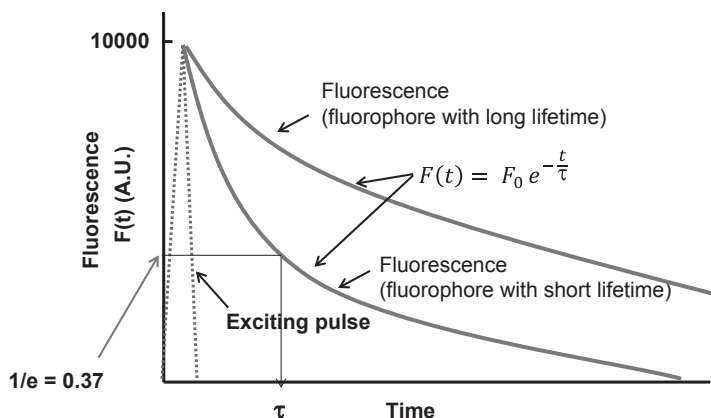


FIGURE 13.5 Fluorescence lifetime. The schematic shows the response of a fluorophore to a typical excitation pulse (blue line). The emission, shown by the green lines, is characterized by exponential decay profiles. The time taken for fluorescence to decay by $1/e$ is the fluorescence lifetime. This corresponds to the average time spent by the fluorophore in its excited state following an excitation pulse.

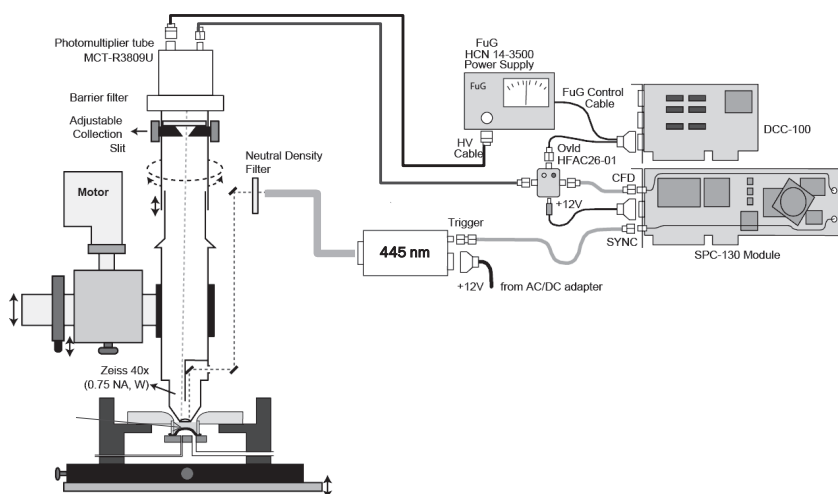


FIGURE 13.6 Schematic of the CSMF with TCSPC. TCSPC approach is a gold standard for an accurate determination of fluorescence lifetime. We have adopted the approach with a custom-made confocal scanning microfluorometer (CSMF) (Maurice and Srinivas 1994; Gupta et al. 2010; Gupta, Chauhan, and Srinivas 2012; Chaiyasan et al. 2017; Niamprem, Srinivas, and Tiyafoonchai 2019) and TCSPC system from Becker & Hickl GmbH.

Srinivas 1994; Gupta et al. 2010; Gupta, Chauhan, and Srinivas 2012; Chaiyasan et al. 2017; Niamprem, Srinivas, and Tiyafoonchai 2019).

Table 13.5 identifies some examples of pO_2 sensing using the TD approach. In the multi-channel scaler approach, the fluorescence lifetime is measured by

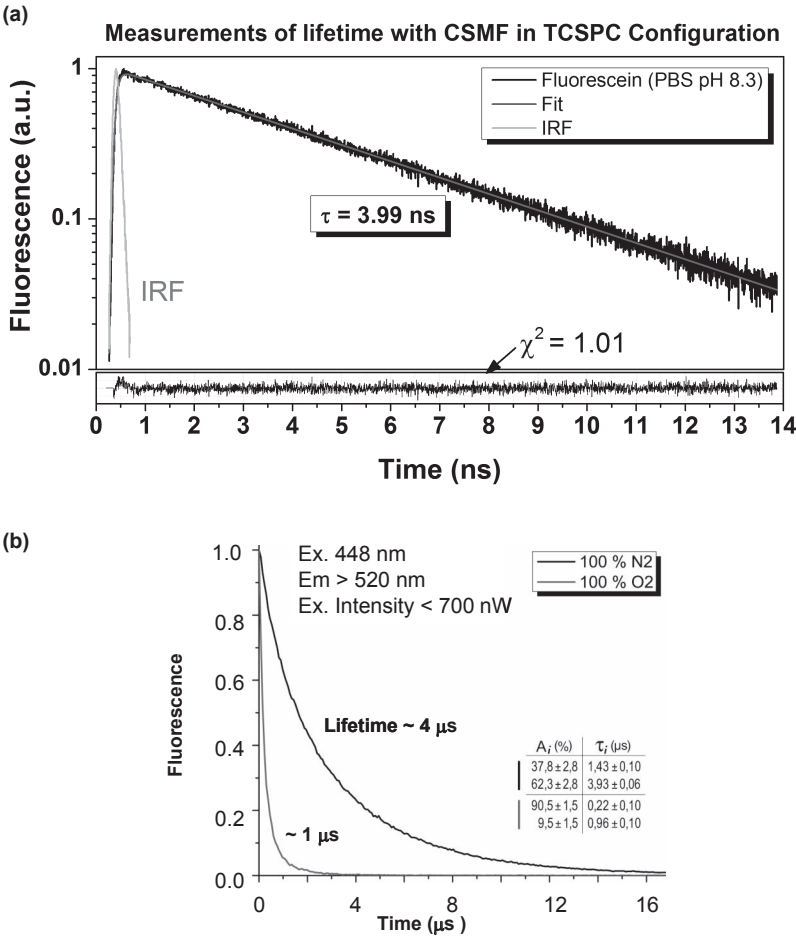


FIGURE 13.7 Fluorescence lifetime measurement by time-correlated single-photon counting (TCSPC). (a) Fluorescence lifetime of fluorescein: the semi-log plot shows the fluorescence decay curve of fluorescein (1 μM) in PBS at pH = 8.0 at 35°C. The measured lifetime of 3.99 ns is close to the values reported in the literature. The instrument response function (IRF) was obtained using Rose Bengal in 5 M KI, which is reported to have a lifetime of 16 ps. (b) Fluorescence decay of Ru-Silica microparticles with TCSPC. The plot shows the fluorescence decay curve for Ru-Phen bound to silica nanoparticles (1 μM) in air and after exposure N₂.

repeated excitation of the sample by pulsed light. In particular, emitted photons for each pulse are binned over a sufficiently large number of time intervals. The number of pulses, pulsing frequency, bin width, and bin interval are all adjusted so that a smooth recording of the decay profile is produced. LEDs and lasers are convenient sources for pulsed excitation. When lifetime is in the range of nanoseconds, the multi-channel scaler technique is not suitable. A popular recourse is to use TCSPC in which the arrival time of the first photon in response to a series of laser

TABLE 13.5
TD and FD Approaches for Measurement of Lifetime of pO₂-Sensitive Dyes

Cells	pO₂-Sensitive Dye	Formulation	Technique	Observations	References
Jurkat T-cells	PtCP (Luxcel Biosciences Cork, Ireland)	Dyes, bound to BS _A , were mixed in PBS to produce a 100 µM stock	Fluorescence microscopy λ _{ex} = 380 nm λ _{em} = 646 nm	τ = 24 µs extracellular, air saturated O ₂ concentration, τ = 31 µs indicating intracellular oxygenation of 71%	O'Riordan et al. (2007a, 2007b)
A549 human lung carcinoma	PtCPK		Fluorescence microscopy λ _{ex} = 399 nm λ _{em} = 767 nm	Demonstrated loading efficiency and intracellular probe localization	
HeLa	PdCPK		Fluorescence microscopy λ _{ex} = 405 nm λ _{em} = 796 nm	PdCPK proved more photostable compared to PtCP	
Squamous and adenocarcinomaesophageal epithelial cells (human)	Ru ²⁺ -tris(4,7-dipyridyl) dichloride hexahydrate (RTDP) (custom synthesis)	0.8 mL of cell lysate in 0.2 mL of 5 mg/mL RTDP at 37°C	Imaging λ _{ex} = 450 nm λ _{em} = 620 nm	τ of normal cells found to be 30 ns shorter than that of cancer cells when exposed to identical O ₂ levels, indicating hypoxia in cancer cells	Sud and Mycek (2009)
Osteosarcoma epithelial (U2OS) cells (human)	Ru(II)-tris(4,7-diphenyl-1,10- phenanthroline) [Ru(dpp)] (Sigma Aldrich, Ireland)	(Ru(dpp)) encapsulated in MTEOS silica nanoparticles	Imaging λ _{ex} = 450 nm λ _{em} = 605 nm	Intracellular pO ₂ in respiring cells ~7.8 ppm Lifetime decreased from 4.4 to 3.3 µs within the dissolved O ₂ concentration range	Korzeniowska et al. (2015)

(Continued)

TABLE 13.5 (Continued)
TD and FD Approaches for Measurement of Lifetime of pO₂-Sensitive Dyes

Cells	pO ₂ -Sensitive Dye	Formulation	Technique	Observations	References
Green alga Chara coralline	Pt(II)-tetra-pentafluorophenyl-porphyrin (PtPFPP)	PtPFPP encapsulated in carboxylated polystyrene microbeads	Two frequency phase modulation $\lambda_{\text{ex}} = 340\text{ nm}$ $\lambda_{\text{em}} = 642\text{ nm}$	With modulated green light excitation, cellular pO ₂ = 250 μM (normal air) With photosynthetically active blue light, pO ₂ increased to 500 μM Technique could be adopted for glucose, [Cu ²⁺] and [Hg ²⁺] measurements	Schmalzlin et al. (2005)
				Lifetime increased by ~100 ns following N ₂ inflow Followed the Stern-Volmer relation with $\tau_0/\tau = 1 + 0.003 [\text{O}_2]$, $\tau_0 = 775\text{ ns}$	Zhong, Urayama, and Mycek (2003)
Epithelial cells (human)	Ru ²⁺ tris(2,2'-bipyridyl) dichloro-ruthenium(II) hexahydrate [RTDP] (Aldrich Chemical Co.)	Stock solution at 6.5 mM	FLIM $\lambda_{\text{ex}} = 460\text{ nm}$ $\lambda_{\text{em}} = 620\text{ nm}$	Difference in lifetime (intra- vs. extracellular) was ~50 ns, yielding a 45.5 μM difference in pO ₂ Cancer cells showed higher intracellular pO ₂ than normal cells by 60 μM	Sud and Mycek (2009)
Normal and cancer cells	Ru ²⁺ tris(2,2'-dipyridyl) dichloride hexahydrate [RTDP]	RTDP dissolved in PBS (0.13 mM)	FLIM $\lambda_{\text{ex}} = 450\text{ nm}$ $\lambda_{\text{em}} = 620\text{ nm}$		

(Continued)

TABLE 13.5 (Continued)
TD and FD Approaches for Measurement of Lifetime of pO₂-Sensitive Dyes

Cells	pO ₂ -Sensitive Dye	Formulation	Technique	Observations	References
Rat C6 glioma	Ru(II)-tris(4,7-diphenyl-1,10-phenanthroline) [Ru(dpp)] (GFS Chemicals, Inc. Columbus, OH)	Ru(dpp) embedded in sol-gel-based PEBBLE ^a sensors	Imaging $\lambda_{\text{ex}} = 450 \text{ nm}$ $\lambda_{\text{em}} = 605 \text{ nm}$	Ratiometric fluorescent determination of O ₂ in gaseous and dissolved phases pO_2 air saturated cells = $7.9 \pm 2.1 \text{ ppm}$ pO_2 nitrogen saturated cells = $6.5 \pm 1.7 \text{ ppm}$	Xu et al. (2001)
Uterine cancer-derived HeLa cells and breast cancer-derived MCF-7 cells (human)	Iridium(III) complex BTpDM ^{1b} – (btp) ₂ Ir(acac) (custom synthesis)		FLIM $\lambda_{\text{ex}} = 485 \text{ nm}$ $\lambda_{\text{em}} = 615 \text{ nm}$	$\tau_{\text{tumor}} > \tau_{\text{extratumor}}$ Demonstrated hypoxia in tumors pO_2 of tumor = 6.1 mmHg pO_2 of extratumor epidermal tissue = 50 mmHg	Yoshihara et al. (2015)
HeLa, A549, Jurkat, SH-SY5Y, C2C12, and PC12 cells	Phosphorescent Pt-coproporphyrin dye (Luxcel Biosciences, Cork, Ireland)	Dye in conjugation with serum albumin 16 (available under the trade name of MitoXpress)	FLIM $\lambda_{\text{ex}} = 340 \text{ nm}$ $\lambda_{\text{em}} = 642 \text{ nm}$	System enabled screening and identification of lead compounds targeting the ETC, mitochondria, Ca ²⁺ channels, ATPases, GPCRs	O’Riorda et al. (2007b)
E-coli cells	FbFP ^c and YFP ^c	A fusion of FbFP and YFP in phosphate buffer	FLIM FbFP, $\lambda_{\text{ex}} = 450 \text{ nm}$ $\lambda_{\text{em}} = 495 \text{ nm}$ YFP, $\lambda_{\text{ex}} = 514 \text{ nm}$ $\lambda_{\text{em}} = 528 \text{ nm}$	τ of FbFP in FluBO ^c = 1.74 ns , Lifetime reduced to 0.99 ns due to FRET	O’Riordan et al. (2007b)

(Continued)

TABLE 13.5 (Continued)
TD and FD Approaches for Measurement of Lifetime of pO₂-Sensitive Dyes

Cells	pO₂-Sensitive Dye	Formulation	Technique	Observations	References
Insect salivary glands	Ruthenium-complex Kr3415 (Sigma-Aldrich, Deisenhofen, Germany)	Dissolved in dimethyl sulfoxide (DMSO)	Simultaneous FLIM/PLIM– $\lambda_{\text{ex}} = 283, 449 \text{ nm}$ $\lambda_{\text{em}} = 638 \text{ nm}$		Jahn, Buschmann, and Hille (2015)
Gastric cancer cell line MKN45	Platinum tetrakis(4-carboxyphenyl)porphyrin [PTCPP] (custom synthesis)	Dissolved in dimethyl sulfoxide (DMSO)	PLIM $\lambda_{\text{ex}} = 405\text{--}515 \text{ nm}$ $\lambda_{\text{em}} = 435\text{--}525 \text{ nm}$	High resolution images of intracellular O ₂ concentration obtained with high repetition rate laser (50 MHz) and confocal microscope Results indicate consumption of O ₂ in the cell	Kurokawa et al. (2015)
Murine renal cells	BTPDM1 ^b	In DMEM/F-12 with FBS	PLIM using TCSPC $\lambda_{\text{ex}} = 470 \text{ nm}$ $\lambda_{\text{em}} = 510 \text{ nm}$	Quantitative evaluation of intracellular O ₂ tension Phosphorescence lifetime of normal kidneys 1.7–1.9 μs corresponds to 50–60 mmHg (6.6%–7.9% O ₂)	Hirakawa et al. (2015)

(Continued)

TABLE 13.5 (Continued)
TD and FD Approaches for Measurement of Lifetime of pO₂-Sensitive Dyes

Cells	pO ₂ -Sensitive Dye	Formulation	Technique	Observations	References
Yeast, Spironucleus cultures, Mammary adenocarcinoma, MCF-7	RuCl ₃ , 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt (Sigma-Aldrich)	Ru coordination complex embedded nanoparticles	2P confocal laser scanning microscopy λ_{ex} = 450 nm λ_{em} = 685 nm Time-resolved epifluorescence microscopy λ_{ex} = 495 nm λ_{em} = 600 nm	Under anaerobic atmosphere Pinhole shifting, τ = 3.19 μ s Epi-phosphorescence-TCSPC, τ = 3.28 μ s, literature τ = 3.88 μ s	Lloyd et al. (2014)
HeLa cells, MDA-MB-231 cells	Pt(II) meso-tetrakis(pentafluorophenyl)porphine (PtTFPP) (custom synthesis)	PtTFPP dissolved into thermal curable poly(perfluoroether) (PFPE)	PLIM λ_{ex} = 532 nm λ_{em} = 600–700 nm	PtTFPP/PFPE film showed better morphology, mitigated phototoxicity of PtTFPP τ_0 = 64 μ s, K_{sv} = 76.9 $\pm$ 4.2 bar ⁻¹	Zhao et al. (2018)

^a Photonic explorer for biomedical use with biologically localized embedding.
^b btp = benzothienylpyridine, acac = acetylacetone. BTP consists of BTPSA (containing an anionic carboxyl group), BTPNH₂ (containing a cationic amino group), and BTPDM1 (containing a cationic dimethylamino group).
^c FbFP = flavin binding fluorescent binding, YFP = yellow fluorescent protein, FluBO= 5Kt341 [bis[(1,10-phenanthroline-4,7-diyl-κN,κN')bis(phenylsulfonate)(2-)] [N-octadecyl-5-(4-(7-phenyl-1,10-phenanthroline-4-yl-κN,κN')phenyl)pentanamide] ruthenium (II) dichloride).

pulses is recorded over a very large number of cycles of excitation. The fluorescence decay profile is reconstructed from the multitude of single photons collected over many cycles. Thus, the TCSPC is based on repetitive, precisely timed registration of single photons of the fluorescence signal. The method is highly accurate, but the sampling rate can be quite limited (Moon, Park, and Won 2020).

O’Riordan et al. (2007a, 2007b) demonstrated transient changes in the mitochondrial activity by monitoring OCR using standard microwell plates with a time-resolved fluorescent reader in different cell suspensions and adherent cell lines, including Jurkat, PC12, A549, HeLa, SHSY5Y, and C2C12. The investigators used a phosphorescent O_2 -sensing probe (MitoXpress™), which was delivered intracellularly by passive liposomal transfer or facilitated endocytosis. The phosphorescence lifetime measurements provide an accurate, real-time, quantitative assessment of pO_2 levels in resting cells and their alterations in response to mitochondrial uncouplers, inhibitors, plasma membrane depolarization, and Ca^{2+} effectors.

Hosny et al. (2012) employed $[Ru(bipy)_3]^{2+}$ to characterize pericellular pO_2 based on measurement of lifetimes using a TCSPC system (Becker & Hickl). In particular, O_2 gradients around chondrocytes in a three-dimensional agarose gel were assayed. Zhao et al. (2018) measured phosphorescence lifetime based on PtTFPP which was incorporated into a non-toxic poly (perfluoroether) (PFPE). The technique was used to measure per-cell O_2 consumption in HeLa and MDA-MB-231 cells based on TCSPC.

Other optical approaches are also available for studies with animal models *in vivo* or tissues (i.e., *ex vivo*). For example, Mik took advantage of the O_2 -sensitive delayed fluorescence of protoporphyrin IX (PpIX), which is found in the mitochondria. PpIX is synthesized inside the organelle from its precursor δ ALA (5-aminolevulinic-acid). It emits delayed fluorescence in response to a green light pulse (Harms et al. 2013; Mik 2013) (Figure 13.8). Since PpIX in its triplet state interacts strongly with O_2 , its lifetime would be increased by O_2 depletion. The lifetime of the delayed fluorescence of PpIX is inversely related to pO_2 by the Stern–Volmer equation (Eq. 13.2). The level of PpIX in the mitochondria can be elevated by exogenous δ ALA (Harms et al. 2013).

We recently established a cuvette-based system with a multi-channel scaler (MCS; SR430; Stanford Research Systems, Inc., CA; 5 ns multi-channel scaler/averager) for a rapid estimate of lifetime (Figures 13.9–13.11) (Sushma et al. 2019). A white LED (Cree XHP70 6V) was pulsed by a current pulse generator (AV-156-A-B) at 12 V DC to generate the excitation pulse train. The pulse generator was programmed to produce a series of constant current pulses of 10 μ s at 2 A in burst mode. The pulse frequency was varied between 100 and 2,000 Hz depending on the number of bins/record chosen in the MCS. The excitation was passed through an interference filter (465 ± 30 nm for Ru-Phen or 530 ± 30 nm for Pd-porphyrin), while the emission was collected through a long pass emission filter having a cut-off at 570 nm. The cuvette could hold the dye in various formulations (gels, encapsulated nanoparticles, and thin film). The emission was detected using a photon-counting photomultiplier (PMT; R3896; Hamamatsu, Inc.). The negative-going pulses output of the PMT below a threshold were counted by the

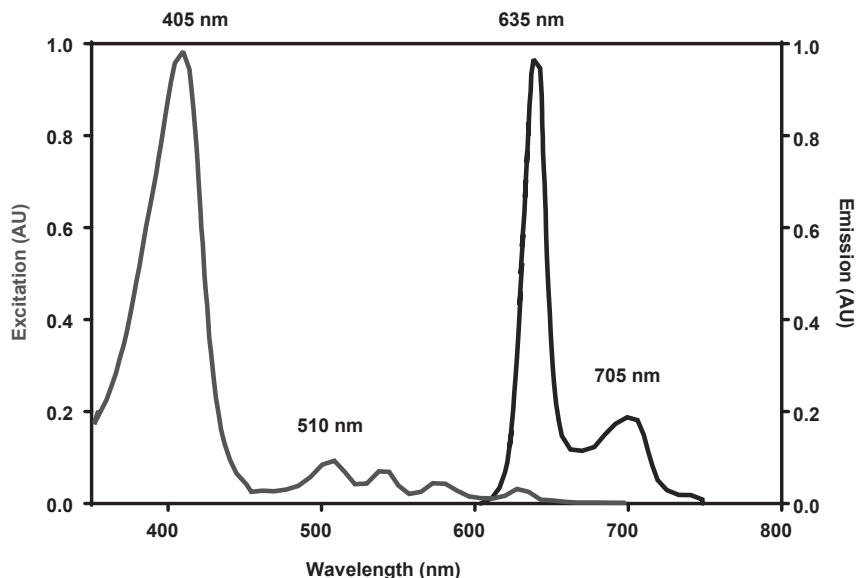


FIGURE 13.8 Absorption and emission spectra of PpIX. The endogenous porphyrin absorbs light at ~405nm and emits fluorescence with a peak centered at 635nm. Quantification of its intensity or lifetime of delayed fluorescence has been employed to assess mitochondrial pO_2 . PpIX is synthesized in the mitochondria from its precursor 5-aminolevulinic acid (δ ALA), which can also be added exogenously.

MCS, which was programmed to have a large number of bins. The photon counts were accumulated over time for each excitation pulse and summed for several pulses (sweeps). The Sync output to trigger the data acquisition in the MCS was generated by a photodiode in line of the excitation. The photon count after a finite number of sweeps vs. bin # gives a representation of fluorescence decay from which lifetime was estimated. By increasing the number of sweeps, it was possible to obtain noise-free measurements. A LabVIEW program was developed to configure the MCS and to calculate fluorescence lifetime by non-linear least squares for off-line calculations. We also incorporated an RLD (rapid lifetime determination) algorithm into the LabVIEW program for a real-time estimation of the lifetime. Depending on the formulation of Ru(II) or Pd-porphyrin, dwell time, record lengths, and records/scan in the MCS were varied as 40–160 ns, 1–8k, 5,000–10,000 pulses, respectively. Since the SR430 is equipped with a trans-impedance amplifier and pulse discriminator, the current output of the PMT was directly coupled to the MCS.

We tested the pO_2 sensitivity of $(Ru(Phen)_3)^{2+}$ and Pd-porphyrin with the MCS system (Sushma et al. 2019). In case of the former, the dye was adsorbed onto silica nanoparticles, which were subsequently mixed in a transparent silicone elastomer dissolved in acetone. The resulting complex was smeared onto a glass coverslip held in the cuvette. The blue excitation was focused on the dye (100 μ m spot).

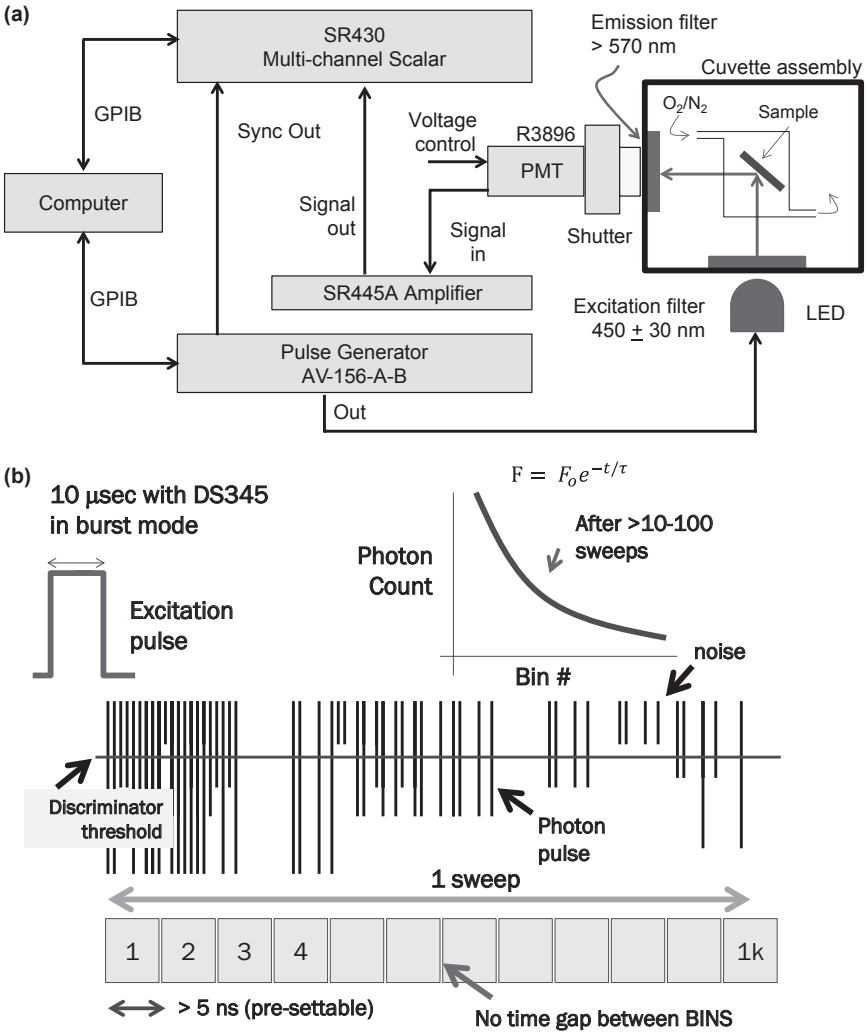


FIGURE 13.9 Measurement of fluorescence lifetime using multichannel scaler (MCS). Application of the TD principle with multiscaler for a rapid estimate of lifetime at different levels of pO_2 . (a) Schematic of the setup with an MCS (SR430; Stanford Research Systems, Inc). (b) Lifetime measurement using an MCS. The negative-going pulse output of the PMT below a threshold was counted by the MCS, which was programmed to have a large number of bins of pre-settable bin width. The photon counts are accumulated over time for each pulse and summed for several excitation pulses (sweeps). The photon counts after a finite number of sweeps vs. bin # give a representation of fluorescence decay from which lifetime was estimated. By increasing the number of sweeps, it was possible to obtain noise-free measurements. (Redrawn from Sushma et al. 2019.)

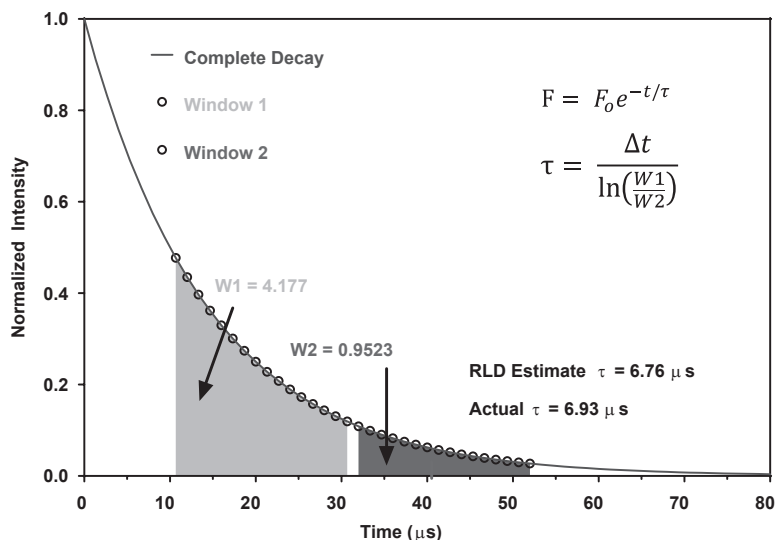


FIGURE 13.10 RLD method for on-the-fly estimates of lifetime. The fluorescence lifetime of this decay is estimated by $\tau = \Delta t / (\ln(W_1/W_2))$ where Δt is the window width, W_1 is the sum of photon counts over Δt in W_1 , and W_2 is the sum of photon counts over Δt in W_2 . The y-axis gives the normalized intensity in photon counts/volts, and the x-axis is the time in μs .

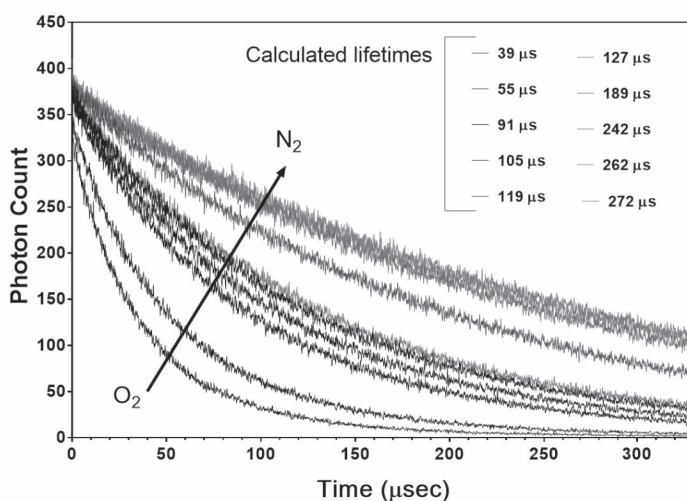


FIGURE 13.11 Fluorescence decay in response to the air to N_2 transition. Ru-Phen sample in the cuvette, which was continuously being exposed to an airstream, was suddenly exposed to a stream of N_2 , and the fluorescence decays were recorded every 2 min until a steady lifetime was obtained. The estimated lifetimes by non-linear least squares were close to the estimates obtained with the RLD approach.

Figure 13.11 shows typical lifetime profiles of the $(\text{Ru}(\text{Phen})_3)^{2+}$ sample in response to changes in pO_2 . In particular, the $(\text{Ru}(\text{Phen})_3)^{2+}$ sample in the cuvette, which was continuously being exposed to an airstream, was suddenly exposed to a stream of N_2 , and the fluorescence decays were recorded every 2 min until a steady lifetime was obtained. The estimated lifetimes by non-linear least squares were close to the estimates obtained with the RLD approach. In its simplest form, the RLD approach utilizes two windows of equal width applied over the decay of a luminophore. The fluorescence intensity of the decay profile is given by $F(t) = F_0 \exp(-t/\tau)$. As shown in Figure 13.10, the fluorescence lifetime of the decay is given by $\tau = \Delta t / (\ln(W_1/W_2))$ where Δt is the window width, W_1 is the sum of photon counts over Δt in W_1 , and W_2 is the sum of photon counts over Δt in W_2 .

13.9 FREQUENCY DOMAIN (FD) APPROACH

The sampling frequency in TD is slow, which can be a concern when sensing rapid changes in pO_2 . On the other hand, the FD approach, in addition to being easy to implement, permits higher sampling frequency (Damale et al. 2019; Sridhar et al. 2019). In the FD, the excitation is modulated as a sine wave (Figure 13.12). The fluorescence emitted by the sample will have a similar waveform but will have been demodulated and phase-shifted from the excitation curve. Both demodulation (M) and phase-shift (ϕ) are determined by the lifetime of the sample emission as shown in Figure 13.13.

A typical implementation of the FD approach involves the use of a single frequency excitation and measurement of phase delay with a lock-in amplifier (Harms et al. 2013; Sridhar et al. 2019). In this approach, it is critical to choose the appropriate frequency of the excitation sine wave for a sensitive pO_2 -dependent phase delay. The optimum frequency which maximizes the phase delay (ϕ) for an incremental change in pO_2 has been investigated by Philip and Carlsson (2003). If τ_1 and τ_2 are lifetimes of the dye (in microseconds) in the presence and absence of O_2 , respectively, the optimal frequency (f^* ; Hz) of excitation is given by $f^* = 10^6 / (2\pi\sqrt{\tau_1\tau_2})$. Thus, for determining f^* , it is imperative to establish estimates of τ_1 and τ_2 , for example, using MCS approach outlined above (Sushma et al. 2019).

Table 13.5 lists exemplary studies that highlight pO_2 sensing by the FD approach. Korzeniowska et al. (2013, 2015) developed silica nanosensors (70 nm) doped with $(\text{Ru}(\text{Phen})_3)^{2+}$ complex. They reported a lifetime of $\sim 1.3 \mu\text{s}$ at physiological pO_2 levels. Importantly, nanoparticles showed no cytotoxicity for 72 hours. Ogurtsov and Papkovsky et al. (2006) tested PtOEPK-PS-based sensor on a porous support with multifrequency phase phosphorometry. Herman et al. (2001) tested an FD approach around a microscope, which employed a modulated LED (370/460 nm; 120 Hz to 250 MHz). Multifrequency phase and modulation responses were collected from cellular areas as small as $10\text{--}15 \mu\text{m}$ in diameter. Fluorescence lifetime data from cells in the range of nanoseconds, microseconds, and millisecond lifetime have been successfully recorded. Their measurements also included pO_2 -sensitive $\text{Ru}(\text{bpy})(2)\text{phe-C}(12)$ in microsecond timescale.

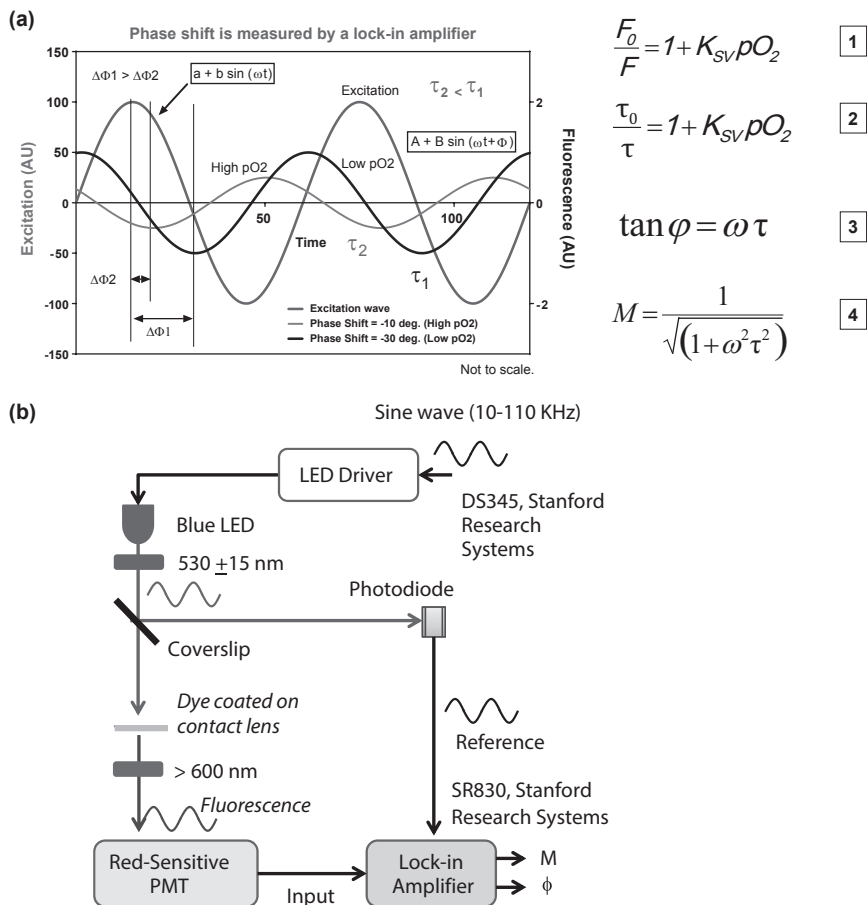


FIGURE 13.12 Frequency domain approach for pO₂ measurement. FD approach is a versatile approach and can be implemented with a lock-in amplifier. (a) Schematic of the FD approach: when excitation is a sine wave, the phase delay (ϕ) in the emission-related to τ by $\tan \phi = \omega \tau$, where $\omega = 2\pi f$ and f is the frequency of the excitation. τ can also be estimated using measured demodulation (M). The optimal frequency f for modulation for enhanced pO₂ measurement (f_{Opt}) (i.e., with the highest S/N ratio) is given by $f_{\text{Opt}} = 1/2\pi(\tau_1\tau_2)$, where τ_1 and τ_2 are lifetimes at maximum and minimum pO₂, respectively. τ_1 and τ_2 can be estimated readily with the multiscaler approach shown in Figure 13.6. (b) Implementation of the FD approach using a lock-in amplifier. Fluorescence lifetime (τ) is related to pO₂ by the Stern–Volmer equations (Eqs. 13.1 and 13.2). In the frequency domain, τ is calculated from the phase delay and demodulation of the emission wave decay in response to pulse excitation. ϕ and M are outputs of the lock-in amplifier. (Redrawn from Sridhar et al. 2019.)

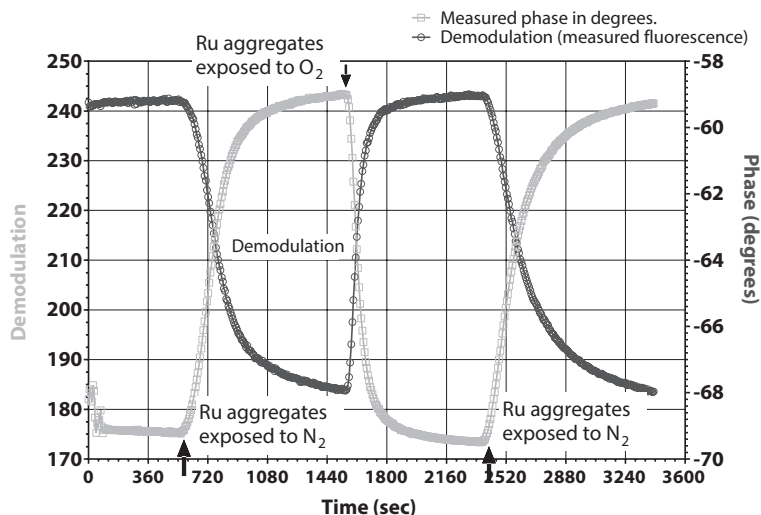


FIGURE 13.13 Changes in ϕ and demodulation in response to N_2 to O_2 transitions. $(Ru(Phen)_3)^{2+}$ response following N_2 to O_2 transition. $(Ru(Phen)_3)^{2+}$ was adsorbed onto silica nanoparticles, and then the particles were mixed in a transparent silicone elastomer dissolved in acetone. This complex was smeared onto a glass coverslip held in an air-tight flow chamber.

13.10 SUMMARY

Nanotoxicity in biomedicine presents a unique set of challenges. The physical and chemical natures of the nanomaterial, as well as the dynamics of the release of drugs/diagnostic agents encapsulated in the nanoparticle system, have the potential to cause nanotoxicity. A large-scale preclinical testing of nanotoxicity requires high-throughput assays. Since the mitochondria play a major role in cellular energetics and other physiological processes (e.g., apoptosis, cytoplasmic Ca^{2+} levels, and reactive oxygen species), their damage would undoubtedly reveal the magnitude and mechanisms underlying nanotoxicity. Both the nanomaterial and active pharmaceutical ingredient (API) may induce mitochondrial dysfunction. Thus, mitochondrial assays are highly relevant to test nanotoxicity. A direct approach to quantify the altered mitochondrial function is to evaluate OCR. In this review, we have highlighted the use of pO_2 -sensitive dyes to measure OCR by fluorescence spectroscopy.

OCR can be assessed in the HTS scale based on the intensity or lifetime of pO_2 -sensitive fluorescent dyes. While the intensity measurements are easy, the technique would yield erroneous measurements when the dye cannot be distributed uniformly in tissues. Moreover, while measuring lifetime, there are two options, viz. TD and FD techniques. Although both techniques are based on the same principle, the FD technique offers high sample acquisition rates and is especially suitable while performing measurements with depth scanning or imaging. We have successfully interfaced a multiscaler to estimate fluorescence lifetime of candidate pO_2 -sensitive probes so that FD approaches can be carried out in an optimal fashion. We have

found the RLD approach to be reliable in the estimates of lifetime. It also enabled the on-the-fly calculation of lifetime in the multiscaler setting. Finally, we have also illustrated the use of TCSPC approach with exemplary measurements.

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14 Current Knowledge on Toxicity of Nanomaterials

Toxicity Assessment and Impact

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14.1 INTRODUCTION

The word “nano-” comes from the Greek language, and it means “dwarf.” Nanomaterials are typically within the size range of 1–100 nm (10^{-9} m) in two or three dimensions.¹ But, in this chapter, we will refer all the materials with any dimension below 1 μ m as nanomaterials. Nanomaterials are characterized by their physicochemical properties like size, surface charge, surface coating, and chemical composition. The unique and enriched electromagnetic, catalytic, pharmacokinetic, and targeting properties as well as physical properties like greater hardness, stiffness, higher thermal stability, yield strength, flexibility, and ductility of nanomaterials make them indispensable for a large number of commercial, technological, and therapeutic applications.^{2,3} As a result of this, nanomaterials are extensively and increasingly used in a wide range of industries ranging from electronics to biotechnology, information technology to structural engineering, energy to biomedical, robotics to defense, paints to environmental engineering, textile to agriculture, food to cosmetic industry, etc.^{4,5} Application of nanotechnology is opening up a new window of opportunities to solve many traditional problems which leads to generation of more and more nanomaterials. The amount of nanomaterial based products is expected to rise up to 58,000 tons by 2020.⁶ Moreover, a host of nanomaterials are generated *via* other anthropogenic activities including combustion of biomass and fossil fuels for transportation, cooking, heating, power generation, waste incineration, smoking, and carrying out occupational activities like welding, mining, building demolition, etc.^{7,8} Besides these, a huge amount of different nanomaterials are being generated *via* different natural processes. Nanomaterials are generated through two processes, viz. either natural or anthropogenic activities. Nanomaterials of natural origin are generated as a result of natural processes like photochemical reactions, volcanic eruptions, forest fires, simple erosion, water evaporation, and by plants and animals, e.g. shedding of skin and hair. Moreover, researchers are collecting extraterritorial nanomaterials in the form of dust from the Moon and Mars. There are also nanomaterials created by microorganisms like magnetotactic bacteria, algae, etc.⁷ Classification of these nanoparticles (NPs) is discussed in the following section.

14.1.1 CLASSIFICATION OF NANOMATERIALS

Nanomaterials can be classified based on their (i) dimensionality, (ii) morphology, (iii) composition, and (iv) uniformity and aggregations (as shown in Figure 14.1).

- i. **Dimensionality:** Nanomaterials are classified based on their dimensions or aspect ratio because dimension plays an important role in determining the physicochemical properties of these nanomaterials.
- **Zero-dimensional (0D) nanomaterials:** Nanomaterials with all three dimensions smaller than 100 nm are called zero-dimensional nanomaterials, e.g. quantum dots and nanodots. The aspect ratio of these particles is approximately 1.
 - **One-dimensional (1D) nanomaterials:** These are nanomaterials with one dimension bigger than 100 nm and in rod shape, e.g. nanowires, nanorods, and carbon nanotubes.

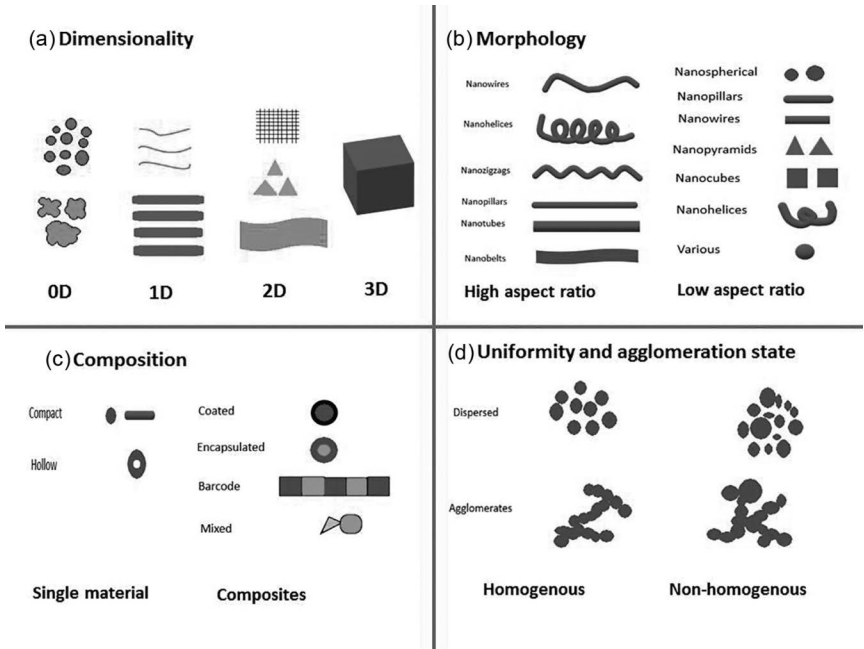


FIGURE 14.1 Classification of nanostructured materials from the points of view of nanostructure dimensions, morphology, composition, uniformity and agglomeration states. (Adopted from refs. 7 and 9).

- **Two-dimensional (2D) nanomaterials:** These are nanomaterials with two dimensions bigger than 100 nm and shaped like thin sheet or film, e.g. graphene, nanofilm, nanolayer, and nanocoating.
 - **Three-dimensional (3D) nanomaterials:** In these nanomaterials, all three dimensions are bigger than 100 nm, e.g. nanocoils, nanoflowers, and liposomes.
- ii. **Morphology:** In this category, nanomaterials are classified based on their aspect ratio (ratio of the breadth to the height), i.e. high aspect ratio and low aspect ratio. Nanomaterials with high aspect ratio include nanotubes, nanocoils, and nanopillars, whereas low aspect ratio nanomaterials are spheres, cubes, oval, etc.
 - iii. **Composition:** Based on the chemical composition, i.e. whether the nanomaterials are composed of single or multiple components, nanomaterials are classified into the following categories: (a) inorganic NPs include all metal and metal oxide NPs, (b) organic NPs include polymeric and biologically compatible NPs, (c) carbon-based NPs include carbon nanotubes, graphene, carbon nanofibers, carbon black, carbon nanofoams, and carbon rods, and (d) organic–inorganic hybrid NPs.⁵
 - iv. **Uniformity and aggregations:** Nanomaterials can exist in dispersed or aggregate states due to their chemistry or surface properties. These materials can be classified based on their uniformity in size as monodispersed and polydispersed materials, whereas based on aggregation, nanomaterials are classified into suspensions and aggregates.

We are constantly exposed to both natural and anthropogenic nanomaterials *via* dust storms, volcanic ashes, and other natural processes. Almost all living organisms are consuming more or less amount of nanomaterials in every walk of life. Though majority of nanomaterials are harmless and their side effect goes unnoticed, occasionally, exposure to these particles are reported to produce some acute or chronic side effects on the host.⁷ In this chapter, we are discussing the impact of these NPs on health and environment with illustrative examples from literature.

14.2 TOXICITY ASSAYS FOR NANOMATERIALS

As the number of NPs is increasing day by day, a wide range of assays are used for evaluating the toxicity of these materials. These assays are classified into two broad categories, i.e. *in vitro* and *in vivo*.

14.2.1 METHODS FOR *IN VITRO* ASSESSMENT OF NANOTOXICITY

Of the two methods for analyzing nanotoxicity, *in vitro* methods are widely preferred owing to their advantages like rapid analysis, cost-effectiveness, and minimal ethical issues. The *in vitro* assays are of the following types.

14.2.1.1 Cytotoxicity Assays

To analyze the cytotoxicity of NPs on a particular cell type, a standard growth curve needs to be obtained for the selected cell type and the cytotoxic effect of NP needs to be analyzed on the basis of growth rates.

14.2.1.1.1 Microculture Tetrazolium Assay

The viability of cells population was studied by Microculture Tetrazolium Assay (MTA). In MTA assay, NP-treated cells were subjected to addition of tetrazolium salts such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) or MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for a period of 2–4 hours at 37°C. The viable cells possessing an active respiratory mitochondrial activity bioreduce MTS or MTT into an insoluble purple formazan product with the help of mitochondrial succinic dehydrogenases. This purple formazan product is solubilized using dimethyl sulfoxide (DMSO) or detergent and then quantified using a visible light spectrophotometer.¹⁰ Ahamed et al. analyzed the viability of mammalian cells as mouse embryonic stem (mES) cells and mouse embryonic fibroblasts (MEF) on exposure to Ag NPs by the MTT assay.¹¹ They showed that Ag NPs significantly decreased the viable cell number with increase in the time of exposure, and Ag-coated NPs showed a higher negative effect on cell viability compared to Ag-uncoated NPs.

14.2.1.1.2 Trypan Blue Exclusion Assay

The cell viability of NP-treated cells was studied in this assay by trypsinizing the treated cells followed by its staining with trypan blue. Trypan blue is a diazo dye which is taken up by dead cells and excluded by viable cells resulting in unstained cells reflecting the total number of viable cells.¹⁰ Bendale et al. evaluated the cytotoxicity of platinum NPs against normal and cancer cells by trypan blue assay.¹² They reported that normal peripheral blood mononucleocyte (PBMC) cells exposed to platinum NPs showed no cytotoxic effect, while cytotoxic effect was clearly demonstrated in cancer cells by trypan blue exclusion assay.

14.2.1.1.3 Clonogenic Assay

This assay studies the survival and proliferation for an extended time period (weeks). The assay involves plating cells at low density and allowed to grow until formation of colonies. The cells may be pre-treated with the NP of interest or treated following cells are plated. The colonies formed are quantified after staining with crystal violet or nuclear stains.¹⁰

14.2.1.1.4 Apoptosis Assay

Programmed cell death or apoptosis has garnered significant use in the assessment of nanotoxicity. Ahamed et al. analyzed the DNA damage by polysaccharide surface-functionalized (coated) and non-functionalized (uncoated) Ag NPs in mammalian cells as mouse embryonic stem (mES) cells and mouse embryonic fibroblasts (MEF), and showed that severe DNA damage could induce the cells

to undergo apoptosis.¹¹ Ahamed et al. provided evidence that silver NP treatment induced apoptosis pathway in larval tissues of *Drosophila melanogaster* as evident from an increased activity of apoptosis markers caspase-3 and caspase-9.¹³ The apoptosis assay encompasses a range of methods as follows.

14.2.1.1.5 DNA Laddering

It assays the DNA damage by characterization of fragmentation by isolating and fluorescently labeling DNA from the NP-exposed cells. This is followed by analyzing the DNA damage by gel electrophoresis.¹⁴ An important characteristic of apoptosis is DNA fragmentation. Gurunathan et al. analyzed the induction of apoptosis following exposure to silver NPs in MDA-MB-231 Human Breast Cancer Cells. They showed that silver NPs exhibited dose-dependent cytotoxicity against MDA-MB-231 cells by activation of lactate dehydrogenase (LDH), caspase-3, and reactive oxygen species (ROS) which ultimately induces apoptosis which is confirmed in the DNA “laddering” pattern exhibited by the treated MDA-MB-231 Human Breast Cancer Cells.¹⁵

14.2.1.1.6 Caspase Assays

This assay measures of zymogen processing to an active enzyme and proteolytic activity. The activation of Caspase-3 results in cell death. The activated Caspase-3 is detected by estimating the cleavage of Caspase-3 substrate linked to a chromophore or fluorophore which absorbs or emits light when separated from the substrate.¹⁴ Baharara et al. studied the induction of apoptosis in HeLa cells by high dose of gold NPs using caspase activation assay. The caspase activity in cells treated with gold NPs was found to be significantly increased indicating apoptosis induction.¹⁶

14.2.1.1.7 Comet Assay

This assay involves analysis and quantification of DNA damage in individual cells. For this, the target cells are embedded in a thin agarose gel on a microscope slide followed by its lysis to remove cellular proteins. The DNA unwinds under alkaline/neutral conditions followed by electrophoresis to allow damaged DNA fragments to migrate from the nucleus. The gel stained with DNA-specific fluorescent dye such as ethidium bromide or propidium iodide is analyzed for fluorescence in the head and tail and the length of the tail. The DNA damage is estimated from the extent of DNA liberated from the head of the comet.¹⁴ Schiavo et al. attempted to analyze the genotoxic effects of ZnO NPs in marine microalgae *Dunaliella tertiolecta*. They reported that ZnO NP-treated algal cells showed comets with undefined head regions and tails of DNA fragments as compared to a compact nucleus having a well-defined head region devoid of tail in untreated algae.¹⁷

14.2.1.1.8 TUNEL Assay

The TUNEL assay analyzes DNA fragmentation by incorporating biotinylated nucleotides conjugated to bromodeoxyuridine (BrdU) at the 3' OH ends of DNA

fragments formed during apoptosis.¹⁴ Li et al. studied the apoptotic effect of gold NPs on AGS gastric carcinoma cells by TUNEL assay and showed that NPs exposure resulted in inducing apoptosis of AGS gastric carcinoma cells in a time-dependent manner.¹⁸ Du et al. evaluated the induction of cardiomyocyte apoptosis by exposure to silica NPs and reported dose-dependent histological and ultra-structural alterations indicating cardiomyocytes destruction with mitochondrial damage. They used TUNEL assay to detect apoptosis and revealed an increasing trend in apoptosis index as dose increased.¹⁹

14.2.1.1.9 *Annexin V and Propidium Iodide (PI)*

Annexin V binding strongly to phosphatidyl serine is widely used for detection of apoptosis. Phosphatidyl serine which is normally excluded from the extracellular side of the plasma membrane, but flips between the inner and the outer sides with the onset of apoptosis. Thus, fluorescent-labeled Annexin V is exploited to identify cellular apoptosis.¹⁴ PI is known to stain the nucleus during the late stage of apoptosis when cell membrane integrity is affected. Baharara et al. studied the induction of apoptosis by a high dose of gold NPs by Annexin V/PI double-labeling flow cytometry. They revealed the potential of gold NPs to induce apoptosis in HeLa cells in a time-dependent manner since the proportion of apoptotic cells (Annexin V positive) increased with the time of exposure. Also, the proportion of cells in late apoptosis (Annexin V positive and PI positive) increased with a rise in the dose of gold NPs.¹⁶

14.2.1.2 **Oxidative Stress Assays**

NP treatment results in the production of reactive oxygen which is detected predominantly by the following.

14.2.1.2.1 *2,7-Dichlorodihydrofluorescein (DCFH) Assay*

In DCFH assay, ROS converts DCFH into a fluorescent product, 2,7-dichlorofluorescein, which is detected using fluorimetry. DCFH as a probe is highly preferred since it is oxidized by many ROS functional groups. DCFH is commonly used probe for quantitative estimation of H_2O_2 along with other members of the hydroperoxide group like tert-butyl hydroperoxide.^{14,20} Wang et al. showed that exposure of ZnO NPs to human pulmonary adenocarcinoma cell line LTP-a-2 induced apoptosis by increasing intracellular ROS levels which was estimated by DCFH assay. The assay showed strong green fluorescence in LTP-a-2 control cells. On the other hand, NP-treated LTP-a-2 cells exhibited blue fluorescence which was found to be strengthened accompanied by the appearance of apoptosis vesicles as the concentration of ZnO NPs increases.²¹

14.2.1.2.2 *Lipid Peroxidation Assay*

Lipid peroxidation is caused by the oxidative stress from ROS. This assay is based on using the products of lipid peroxidation cycle as oxidative stress markers after NP treatment. The products of lipid peroxidation, lipid hydroperoxides, and aldehydes like malondialdehyde (MDA) can be measured by thiobarbituric acid

reactive substances (TBARS) assay where MDA combines with thiobarbituric acid (TBA) resulting in a fluorescent adduct which can be detected at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.²² Govender et al. attempted to understand the apoptosis induction in human lung carcinoma cell line on silver NP treatment. For this, they analyzed the cellular oxidative status by lipid peroxidation assay and found that lipid peroxidation was significantly fivefold higher in cells exposed to silver NP.²³

14.2.2 METHODS FOR *IN VIVO* ASSESSMENT OF NANOTOXICITY

Animal models such as mice and rat are routinely used to study *in vivo* toxicity of NPs comprising a range of analysis methods such as biodistribution, clearance, serum chemistry, and histopathology.

14.2.2.1 Biodistribution

The physiochemical characteristics of NPs determine their biodistribution and nanotoxicity. This is the most common technique to evaluate biodistribution of nanomaterials in animal models. The presence of nanomaterials in vital organs or tissues such as lung, liver, kidney, heart, spleen, pancreas, brain, fat, and muscle is estimated following administration of nanomaterials. Then, the distribution of NPs can be tracked in these organs using physical detection methods.²⁴ Balogh et al. studied *in vivo* distribution of gold composite NPs in mouse tumor models by instrumental neutron activation analysis and showed that composite nanodevices (CNDs) on basis of their size and/or surface charge could selectively target specific organs without any specific targeting moieties on its surface.²⁵ Guo et al. studied the synergistic effects of silica NPs (Si NPs) and CdCl₂ on their *in vivo* distribution in mice by inductively coupled plasma-atomic emission spectrometry. They showed that co-exposure of SiNPs and CdCl₂ increased the accumulation of Cd in the liver inducing hepatic oxidative stress.²⁶

14.2.2.2 Clearance

The clearance of NPs is studied by examining the excretion and metabolism of NPs at a specific time interval following NP treatment. Li et al. analyzed the biodistribution of PEGylated poly(lactic-co-glycolic acid) (PEG-PLGA) NPs following intravenous administration for a period of 24 hours in rats. The blood clearance studies after the intravenous administration of bovine serum albumin (BSA)-loaded NPs revealed that PEG-PLGA NPs initially had higher blood circulating levels compared to PLGA NPs. The levels of BSA in blood following 1 hour of intravenous injection of PEG-PLGA NPs were found to be 12-fold higher than those in the case of PLGA NPs. BSA loaded in PEG-PLGA NPs exhibited significantly delayed blood clearance over PLGA NPs resulting in blood-associated BSA being higher after 24 hours as compared to PLGA NPs. The distribution profile of BSA loaded in NPs showed that after 3 hours of intravenous administration, BSA loaded in PLGA NPs were distributed to the liver, kidney, spleen, and lungs with their blood levels being significantly low. It was observed

that BSA loaded in PLGA NPs was gradually eliminated from all tissues after 12 hours after intravenous injection. On the other hand, BSA loaded in PEG-PLGA NPs exhibited a differential extent in its transport from blood to tissues. The level of BSA loaded in PEG-PLGA NPs in plasma was significantly high as compared to that for PLGA NPs at the same time interval following intravenous injection. Along with this, their transport to the liver and kidney were found to be markedly limited.²⁷

14.2.2.3 Hematology and Serum Chemistry

The analysis of changes in serum chemistry and hematology following NP treatment is used to assess nanotoxicity. Serum chemistry studies involve determination of the following parameters: blood urea nitrogen, creatinine, glucose, total protein, albumin, globulin, albumin to globulin ratio, sorbitol dehydrogenase (SDH), alanine aminotransferase, alkaline phosphatase, and creatine kinase (CK). Hematological analysis involves determination of the following parameters: red blood cell count, hemoglobin, packed cell volume (PCV), mean red cell volume, mean red cell hemoglobin, mean red cell hemoglobin concentration, white blood cell count, differential white blood cell count, and platelet count. Baker et al. used hematology and serum chemistry analysis to understand the toxicity of C60 fullerene NPs in male rats. The exposure of rats to C60 fullerenes NPs resulted in decrease in red blood cells, hemoglobin, and pack cell volume. The NP exposure caused decrease in monocytes and platelets with an increase in eosinophils. Blood SDH, a hepatic enzyme, was decreased in NP-exposed rats indicating NP exposure-related hepatic effects.²⁸ Lei et al. studied serum clinical biochemistry parameters to characterize the toxicity induced by high doses of nanocopper. Rats exposed to high-dose nanocopper showed an increase in the serum plasma enzyme aspartate aminotransferase and alanine aminotransferase along with plasma metabolite bilirubin indicating significant liver damage. Further, an increase in blood urea nitrogen and creatinine showed dysfunction in renal glomerular filtration.²⁹

14.2.2.4 Histopathology

Nanotoxicity is also determined by histopathological examination of NP-exposed organs such as eyes, brain, liver, kidneys, lung, heart, and spleen. Lei et al. carried out histopathological examination to characterize the hepato- and nephrotoxicity induced by nanocopper. The comparison of hematoxylin and eosin liver sections obtained from nanocopper-exposed and control groups showed scattered dot hepatocytic necrosis in rats of 200 mg/kg/d group, while no overt toxicity indications were observed in mid- or lower dose groups. Necrosis of renal proximal tubule involving most neurons and cellular fragments in tubule lumen with deposition of orange crystal matter indicated nanotoxicity to renal tubules.²⁹ Baker et al. applied histopathological analysis of the brain, eyes, liver, kidneys, spleen, heart, lungs, large and small intestines, testes, epididymides, urinary bladder, lymph nodes (bronchial, mandibular, mediastinal, mesenteric), larynx, and nose to understand the toxicity of C60 fullerene NPs in male rats. They

showed that exposure of C60 fullerene NPs over a period of 10 days did not reveal any exposure-related microscopic lesions.²⁸ Zhu et al. studied the pulmonary responses to nano-ferric oxides in rats by histopathological analysis. The follicular lymphoid hyperplasia with inflammatory cells aggregated around bronchia was found following nano-ferric oxide exposure. NP treatment resulted in thickening of alveolar walls indicating fibrosis formation. The macrophage-phagocytosed particles were found to be evident in the pulmonary alveoli. The inflammatory cells like neutrophils, lymphocytes, and eosinophils were found inside the pulmonary alveolus. The inflammatory responses were found to be still extended at 30 days post-instillation presenting as the severe follicular hyperplasia of the lymph node, capillary hyperemia, alveolar lipoproteinosis, and emphysema.³⁰

14.3 IMPACT OF NANOMATERIALS ON HEALTH AND ENVIRONMENT

14.3.1 IMPACT OF NANOMATERIALS ON HEALTH

The effects of both natural and anthropogenic NPs on human health are discussed in the following section (shown in Figure 14.2).

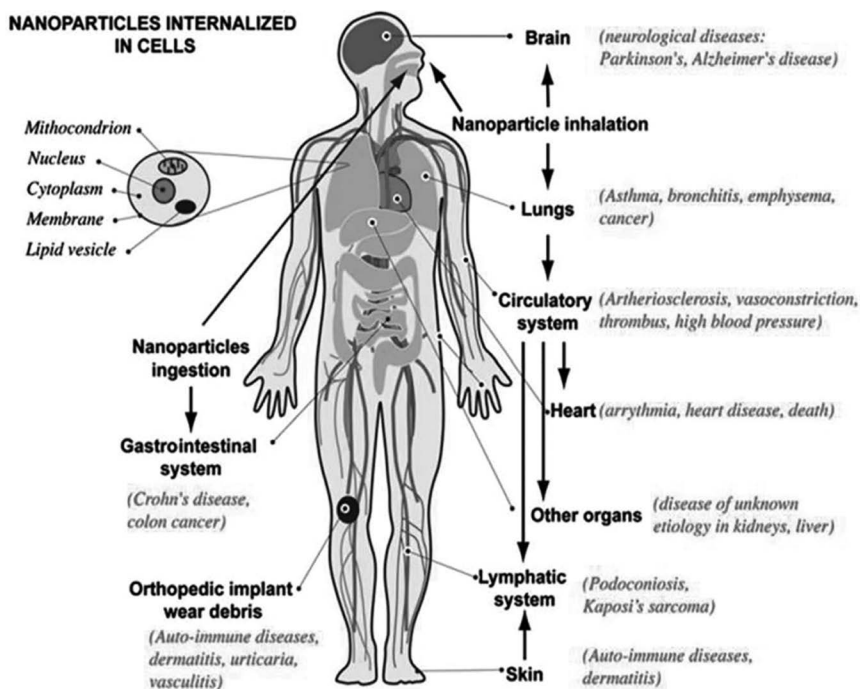


FIGURE 14.2 Schematics of human body with pathways of exposure to nanoparticles, affected organs, and associated diseases from epidemiological, *in vivo*, and *in vitro* studies.⁷

14.3.1.1 Impact of Natural Nanomaterials on Health

Humans are susceptible to some acute and chronic effects due to exposure to a number of different types of nanomaterials in their day-to-day life. Exposure to airborne dust particles and nanomaterials generated from forest fire cause respiratory diseases.

Terrestrial dust storms are probably the largest source of environmental NPs and carry both natural NPs from mostly mines, deserts, etc. and manmade NPs from anthropogenic activities like burning of fossil fuels and biomass.

Airborne nanomaterials were reported to be responsible for asthma and emphysema. The presence of iron or other metals in the dust particles generates ROS which in turn create scar in the lungs.³¹ Extraterrestrial dusts from the space, Moon, and Mars missions were reported to increase respiratory diseases in astronauts.³²

Nanomaterials generated during forest fires were reported to deteriorate the health condition of patients with preexisting cardiopulmonary diseases during smoke saga.³³ Volcanic ash and gases release a huge amount of particulate matter of nano- and micrometer dimensions to the atmosphere.³¹ In the short term, these ash and gases adversely affect the respiratory system, eye, and skin of the host. In Africa, Mediterranean and Central America, due to the long-term exposure to volcanic soils, the barefoot agriculture populations are reported to suffer from diseases of lympho-endothelial origin including podoconiosis^{34–36} and Kaposi's sarcoma.^{33,37} In podoconiosis, the lower limb of the patient swells irreversibly due to failure of lymphatic drainage system resulting from long-term absorption of micro- and nanomaterials from soil by the skin of lower limbs. It is found in almost 10% population of volcanic tropics. Kaposi's sarcoma is a type of cancer related to herpesvirus infection which affects the blood and lymph vessels of the patients. It is predominant in the parts of world containing volcanic soils.³³

Evaporation of water from ocean or other water bodies leads to generation of aerosols containing a large amount of micro- and nanometer-sized particulate matters. No adverse health effect is reported from these materials. On the contrary, these materials are found to be beneficial for human health.³⁸

14.3.1.2 Impact of Anthropogenic Nanomaterials on Health

Humans have created a wide variety of nanomaterials by processes like cooking, automobile industry, burning fossil fuels and biomass, smoking, chemical manufacturing, etc. These manmade nanomaterials are used in cosmetics and wellness products, structural materials, pharmaceuticals, sporting items, textile industry, agriculture, defense, and aerospace industries. We are discussing the adverse effects of major manmade NPs in the following section.

In the urban area, diesel and automobile exhausts are the major contributors of atmospheric pollution. These exhausts release a huge amount of micro- and nanomaterials to the atmosphere.³⁹ Carbon nanotubes and fibers are reported to be the by-products of diesel combustion.⁴⁰ The aspect ratio of these fibers is similar to the asbestos retained in the lungs, suggesting the potential risk of nanomaterials.⁴¹ These nanomaterials are found to be responsible for a variety of lung

diseases. Automobile exhaust-linked atmospheric pollution is responsible for increase in cardiopulmonary disease-related deaths in the population living near the major roads.^{42,43} Childhood cancer is found to be strongly linked with exposure to oil-based combustion gases and engine exhaust.⁴⁴

As per the Environmental Protection Agency (EPA), indoor air can be as much as ten times more polluted than air of non-cooking hours. Burning of solid fuels and poor ventilated stoves lead to the death of a large number of people in Asia and Africa. World Health Organization reported 1.6×10^6 people die annually, and out of which, more than 50% is children of age below five years (WHO report)⁴⁵.

Cigarette smoke is composed of a wide range of NPs and toxic chemical compounds.⁴⁶ Smoking of cigarettes leads to increase in respiratory illness, cardiovascular problems, and a number of different types of cancers including cancer in the lungs and in oral and nasal cavities.⁴⁷ It leads to even genetic mutation.⁴⁸

Building demolition materials contain a large number of micro- and nanoparticulate matters composed of respirable asbestos fibers, lead, glass, wood, paper, and other toxic materials.⁴⁹ These materials can enhance respiratory problems.⁵⁰ However, Long-term impact of these materials is not known yet.

Nanomaterials are extensively used in cosmetics and other personal care products. Nanostructured black soot and mineral powder have been used in these products from ancient times due their ability to penetrate the skin and deliver nutrients deep inside the layers of skin,⁵¹ conceal wrinkles.⁵² Some of these nanomaterials like functionalized fullerenes are claimed to have radical scavenging properties.⁵² Titanium dioxide NPs with diameters larger than 100 nm is reported to be biologically inert,⁵³ and hence, has been used extensively in white pigment, food colorant, sunscreens, and cosmetic creams.⁵⁴ However, recent studies revealed the adverse effects of titanium dioxide NPs.^{55–59}

Due to the antibacterial/antifungal properties of silver NPs, these particles are used in air and hand sanitizer sprays, clothes and garments, detergent, soap, shampoo, toothpaste, air filters, coatings of refrigerators, vacuum cleaners, food storage containers, cellular phones, personal protection equipment kits, etc.⁵² Besides these, a host of NPs have been used in a variety of cosmetics and other personal care products. Health effects of all these NPs used in consumer care products are not known properly. But, recent studies demonstrated the toxicity of these NPs which were regarded as safe earlier. Silver NPs are reported to be more toxic than asbestos.⁴⁰ The side effects of nanomaterials on human health are tabulated in Table 14.1.

14.3.2 IMPACT OF NANOMATERIALS ON THE ENVIRONMENT

With increase in the production and usage of NPs, the amount of NPs being released into the environment is going to increase.^{86–90} NPs are basically released into the environment during usages, recycling, and disposal.^{88,89,91,92} These NPs reveal their toxicity on environmental flora and fauna based on their dose or concentration, particles size, and surface chemistry.^{89,90,92} Ni, Ag, and Zn NPs are reported to kill pathogenic bacteria such as *Staphylococcus aureus*.^{93–95} Since

TABLE 14.1
Side Effects of Nanomaterials on Human Health

Sl. No.	Nanoparticles	Applications/Availability	Side Effects
1	Beryllium	Electrical and electronic parts, and plastic industries	Inhalation can damage the lungs and cause acute pneumonia like syndrome, hypersensitivity, and allergic reaction. In chronic beryllium disease, white blood cells accumulate around absorbed beryllium particles and form granulomas, leading to anorexia, weight loss, cyanosis of the extremities, and heart enlargement. Long-term exposure to beryllium may cause cancer in animals and humans ^{60,61}
2	Lead	Air, household dust, food, drinking water, in batteries, metals, paints, printing industries	High levels of exposure to lead and its compounds can cause serious disability. Inhaled or ingested lead circulates in the blood and is deposited in bone and other tissues. Most of the inhaled lead gets distributed into different organs via blood circulation which leads to intoxications like impairment of mental functions, visual motor performance, memory, and attention span, as well as anemia, fatigue, lack of appetite, abdominal pain, kidney disease, etc. ⁶²
3	Cobalt	Mining	Exposure to cobalt leads to asthma, acute illness, fever, anorexia, malaise, and difficulty breathing, resembling a viral illness, and interstitial pneumonitis ^{60,63}
4	Cadmium	Batteries, pigments, metal coatings, plastics, burning of fossil fuels and cigarettes	Cadmium from industrial and consumer waste accumulates in soil and enters into humans and animals through food chain. High dose of inhaled cadmium causes severe lung irritation, nausea, and vomiting. Long-term exposure even at low dosage causes lung emphysema, breakdown of the immune system and central nervous system, and liver damage, and even cause cancer in humans ^{62,64}
5	Aluminum	Food, water, pharmaceuticals and metal industries	Exposure to aluminum leads to host diseases like anemia, bone disease, and dementia, dialysis encephalopathy, Parkinson's dementia, and Alzheimer's disease. Experimental rats receiving subcutaneous injection of aluminum glutamate showed pathological signs similar to human Alzheimer's disease ^{62,65-68}

(Continued)

TABLE 14.1 (Continued)
Side Effects of Nanomaterials on Human Health

Sl. No.	Nanoparticles	Applications/Availability	Side Effects
6	Nickel and chromium	stainless steel and other metal industries	Inhalation of nickel dust and fumes leads to sinus and lung cancers. Exposure to chromium also leads to other cancers ⁶³
7	Manganese	Water, mining and welding	Manganese is an essential nutrient, but high doses lead to neurological disorders like Parkinson's disease ^{69,70}
8	Iron	Mine, dust, industries	Exposure to increased level of iron can cause cancers like adenocarcinomas, colorectal tumors, hepatomas, mammary tumors, mesothelioma, renal tubular cell carcinomas, and sarcomas. Iron accumulation in the brain may cause neurological diseases including Parkinson's disease and Alzheimer's disease. Iron dust inhalation may cause a respiratory disease called pneumoconiosis ^{63,71,72}
9	Organic dust	Animals and/or plants, and their products	Organic dust from these various sources irritates the upper respiratory system, eyes, and skin, and cause bronchitis ⁶²
10	Silica	Sand and granite quarries, dust	Exposure to silica or silicon dioxide can cause silicosis, a disabling pulmonary fibrosis, which may lead to lung cancer. Besides this, exposure to silica is responsible for autoimmune diseases including scleroderma, rheumatoid arthritis, and systemic lupus erythematosus ^{62,73}
11	Coal and coal ash	Coal mine and coal burning	Coal dusts cause pneumoconiosis in coal miners, and death toll increases due to heart and respiratory diseases, lung, esophageal, and liver cancer ^{74,75}
12	Asbestos	Asbestos industries	Exposure to asbestos leads to lung cancers and increases the risk of autoimmune diseases like systemic lupus erythematosus, scleroderma, and rheumatoid arthritis ^{62,73,76-78}

(Continued)

TABLE 14.1 (Continued)
Side Effects of Nanomaterials on Human Health

Sl. No.	Nanoparticles	Applications/Availability	Side Effects
13	Polymer fumes	Polymer industries	Exposure to polytetrafluoroethylene or Teflon and other polymer fumes develops influenza-like syndrome or polymer fume fever. Prolonged exposure leads to chest pain, fever, chills, sweating, nausea, and headache. Severe toxic effects, such as pulmonary edema, pneumonitis, and death, are also possible ^{79,80}
14	TiO ₂	Building, engineering, agriculture, paints, health care and wellness products	Regular consumption of TiO ₂ NPs even at small doses can affect internal organs of animal body, which can increase the risk of developing many diseases including tumours ^{81,82}
15	ZnO	Health care and wellness products	<i>In vitro</i> experiment demonstrates that ZnO nanoparticles (NPs) are reported to induce cell death by increasing ROS generation and DNA damages ⁸²
16	Ag	Electronics, textile, health care, and wellness products	Ag NPs demonstrate varying degrees of toxicity: from no toxicity to development of inflammatory lesions in the lungs in different studies depending upon NPs' size, dose, animal model, and route of administration. Further studies are required to evaluate the toxicity of these NPs in human ⁸³
17	Fe ₃ O ₄	Health care and wellness products	<i>In vivo</i> studies demonstrated that Fe ₃ O ₄ NPs are fairly biocompatible, and no adverse effects were observed in animal models ^{84,85}

bacteria is an essential organism in our ecosystem and it helps in decomposing organic matters and base for many aquatic and terrestrial food web, loss of bacterial community adversely affects the ecosystem. Ag, ZnO, and TiO₂ NPs are reported to have a toxic effect on *Escherichia coli*.^{93–95}

14.3.3 FATE OF NANOMATERIALS

Nanomaterials enter into mammalian system through the routes like respiratory tract, gastrointestinal (GI) tract, skin, worn-out debris from implants, and even through intravenous injection. Airborne NPs enter into the body through the respiratory tract and get deposited in the nose, pharynx, and lungs.^{96,97} The internal surface area of the lung is between 75–140 m², and it contains almost 300 × 10⁶ alveoli.⁹⁸ Spherical particles of diameter smaller than 10 μm can reach up to the surfaces where gases are exchanged, whereas larger particles deposit in the respiratory tract.^{96,98} Fibers can deposit deep in the lungs and upper airways depending upon their length.⁹⁸ After getting absorbed in the lung epithelium, NPs reach cells in bone marrow, lymph nodes, spleen, and heart *via* blood and lymph.^{86,99} These particles can reach even the central nervous system and ganglia.¹⁰⁰ Inhaled NPs cause respiratory and cardiovascular problems and increase the morbidity and mortality in the susceptible group of population.⁸⁶ The impact of nanomaterials on peripheral nervous system is shown in Figure 14.3.

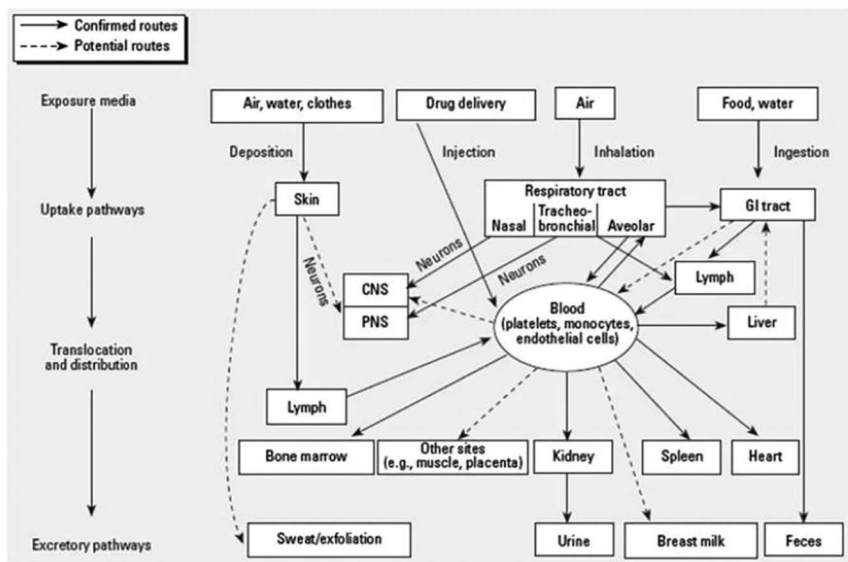


FIGURE 14.3 Different pathways of nanoparticles: PNS, peripheral nervous system. Although many uptake and translocation routes have been demonstrated in literature, others still are hypothetical and need to be investigated.⁸⁶ (Reproduced with permission from National Institute of Environmental Health Sciences.)

NPs can enter into the GI tract through food, cosmetics, drugs, drug delivery devices, and through inhalation.^{86,99,101,102} Biodegradable NPs in oral vaccine undergo proteolysis.¹⁰³ The most common food-grade ingested engineered nanomaterials are TiO_2 (only part of the product is in the nanoscale), SiO_2 (silicon dioxide), iron oxides (Fe_2O_3), zinc oxide (ZnO), and silver (Ag).¹⁰⁴ These NPs may translocate to different organs of the body *via* systemic circulation.¹⁰⁵ There are limited studies available on the toxicity of NPs following oral administration. *In vivo* experiment on mice demonstrated that ingested nanocopper causes pathological damage to the liver, kidney, and spleen.¹⁰⁶ Wang et al. reported the biodistribution of orally administered TiO_2 NPs in the liver, spleen, kidneys, and lung tissues of adult mice indicating the translocation of TiO_2 particles to other tissues and organs following uptake by the GI tract.¹⁰⁷ In another study, Muller and Keck reported the possibility of recrystallization of supersaturated nanosuspension in the GI tract.¹⁰⁸

The skin is the largest organ of human body. It comes into contact with a large number of NPs through occupational and non-occupational means.¹⁰⁰ Occupational exposures include exposures during manufacturing of solvents, pesticides, or pharmaceuticals and their applications, long-term exposure to volcanic soils, and the barefoot agriculture populations, whereas non-occupational exposures are like use of cosmetics, topical creams, and other drug treatments.^{86,99,106} The outer layer of the skin consists of a layer of dead keratinized cells (stratum corneum) of 10 μm thickness. So, it is difficult for NPs to pass this layer and enter into our body.¹⁰⁹ However, NPs or even microscopic particles can easily enter through damaged or broken skin and reach the lymphatic system and regional lymph.¹⁰⁰ Through the lymphatic system, NPs can translocate to systemic vasculature and other organs of human body.⁸⁶

Assessment of the fate of NPs following intravenous injections is necessary, because a lot of nanodrug delivery systems have been prepared for delivering through this route. Jaiswal et al. reported biocompatibility and biodistribution study of Fe_3O_4 -based nanohydrogel made up of chitosan and poly(N-isopropylacrylamide) in Swiss mice following intravenous injection. The results showed that the hydrogel is fairly biocompatible and a significant amount of Fe_3O_4 NPs were found to accumulate in the lungs, liver, and spleen.⁸⁵ In another study, Gogoi et al. demonstrated that magnetic liposomes administered intratumorally following repeated round of hyperthermia deposit in the spleen, lungs, and heart due to the translocation.¹¹⁰ These studies showed the fate of different nanomaterials in different animal models. However, depending upon the type of NPs and their properties like size, surface coating, and zeta potential, biodistribution of these nanomaterials may vary. So, each nanoformulation aimed for human application needs to be evaluated for their bioavailability.

NPs released into the atmosphere undergo dilution and transformation *via* advection and dispersion into the surrounding environment. The ultimate fate of the NPs depends on mechanisms like removal through human inhalation, skin, ingestion, wet and dry depositions, coagulation, advection, and turbulent mixing and evaporation of volatile particles.¹¹¹ Once the airborne NPs enter our body,

these NPs also get distributed in different organs *via* translocation process as discussed earlier. Depending upon the type of NPs, the atmospheric lifetime of particles varies due to the varying effects of deposition, coagulation, evaporation, and transformation processes acting upon them. These NPs tend to aggregate and deposit on nearby surfaces when they reach a threshold particles number concentration (PNC), and PNC can vary depending upon the type of NPs. Dry deposition is influenced by physical processes like Brownian diffusion, interception, inertial and turbulent impaction, gravitational settling, and on water surfaces by phoretic (i.e., drift) effects.¹¹² In wet deposition, NPs are washed by water. The potential routes of exposure, uptake, distribution, and degradation of NPs in the environment are shown in Figure 14.4.

With increased used in consumer products like sunscreen and cosmetics, a large number of nanomaterials are finally washed away and end up in the aquatic environments. These NPs are found to be toxic to different aquatic species like fish, daphnia, and other unicellular organisms.¹¹³ These NPs demonstrate varying degrees of adverse effects on aquatic lives as the large surface area of these NPs generates ROS which are harmful and can seriously damage the proteins, DNA, and membrane of aquatic lives.^{114,115} Nanotoxicity of ENPs of nickel, copper, silver, and aluminum was reported to evaluate in aquatic species like algal species, dolphins, and zebra fish. The results showed that silver and copper NPs had hazardous effects on all these organisms.¹¹⁶ In another study, risk assessment of NPs was evaluated in zebra fish due to its high degree of homology with human.¹¹⁷ Silica NPs were reported to be non-toxic to the embryos of zebra fish,¹¹⁸ whereas Ag NPs were reported to have deleterious effects on aquatic life¹¹⁷ and to cause a number of changes at cellular level including DNA damage which lead to apoptosis of hepatic cells.¹¹⁹

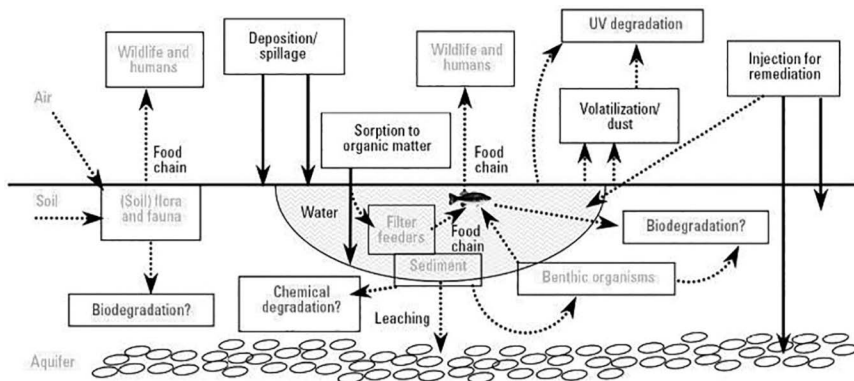


FIGURE 14.4 Routes of exposure, uptake, distribution, and degradation of NSPs in the environment. Solid lines indicate routes that have been demonstrated in the laboratory or field or that are currently in use (remediation). Magenta lettering indicates possible degradation routes, and blue lettering indicates possible sinks and sources of NSPs.⁸⁶ (Reproduced with permission from National Institute of Environmental Health Sciences.)

Similarly, TiO_2 NPs were also reported to be toxic to zebra fish^{120,121} and rainbow trout.¹²² These TiO_2 NPs were reported to impair the activity of Na^+/K^+ -ATPase in the intestine, gills, and enzyme activity in the brain of the rainbow trout. Water-soluble fullerene was found to be toxic to aquatic lives like *Daphnia magna*, *Hyalella azteca*, and marine harpacticoid copepod. Upon exposure to fullerene, the molting and offspring production process of daphnia was delayed significantly.¹²³ Aruoja et al. reported that NPs such as ZnO , CuO , and TiO_2 are toxic for algae. The degree of toxicity of these NPs to the aquatic lives depends on factors like size, type of NPs, charge, and the species which is being exposed.¹²⁴

14.3.3.1 Effect on Soil Environment

It is noteworthy to evaluate the impact of NPs on the lives present on the land mass especially plants, earthworms, nematodes, and soil enzyme activities.¹²⁵ The NPs have different effects on plants and other organisms depending upon factors like plant species, NPs, and environmental factors. The effects of different nanomaterials on plants and microbes are shown in Table 14.2.

14.3.4 MECHANISM OF NANOMATERIAL TOXICITY

Upon exposure to cells, NPs are internalized *via* endocytosis and phagocytosis processes. Uptake of smaller particles happens by endocytosis process, whereas it occurs for larger particles through phagocytosis and it is restricted to specialist cells only (the major phagocytes including monocytes, macrophages, dendritic cells, and neutrophils).¹⁵⁹ Key factors in hazard evaluation are generally chemical composition, dose, and exposure route of particles. However, toxicity of NPs depends on factors like particles size, surface properties, quantum effect, nanostructures, concentration, and self-assembly.⁸⁶

The possible mechanisms of NPs toxicity are injury of epithelial tissue,¹⁶⁰ inflammation, oxidative stress response,^{161–165} and allergy¹⁶⁰ (as shown in Figure 14.5). In the early stage of cellular injury, NPs prompt generation of ROS and oxidative stress. *In vitro* experimental results demonstrated that NPs are responsible for induction of oxidative stress in keratinocytes, monocytes/macrophages, endothelial cells, and blood monocytes cells.^{163,166–170} NPs-induced oxidative stress involves mitochondrial respiration, mitochondrial apoptosis, activation of NADPH oxidase system, alteration of calcium homeostasis, and depletion of antioxidant enzyme, and these lead to tissue injury.¹⁷¹ NP-induced ROS response activates cell signaling pathways, inflammatory cytokine and chemokine expressions, as well as lead to specific transcription factor activation which are closely associated with transcription of genes involved in inflammation, genotoxicity, fibrosis, and cancer.^{163–165,168,172} Thus, the exposure to NPs leads to changes in pathological events *via* ROS generation. Exposure to NPs like carbon nanotubes, carbon black, fullerenes, silica, and metal or metal oxide NPs is reported to cause pulmonary inflammation and/or fibrosis after intratracheal installation.^{173–177}

Studies reported that exposure to some of NPs at various doses leads to increase in pro-inflammatory gene expression.^{166,167,173,178,179} Subsequent cascading

TABLE 14.2
Effect of Different Nanoparticles on Germination and Growth of Plants or Microbes

Sl. No.	Nanoparticles	Crop/Plant/Microorganism	Effect on Crop/Plant/Microorganism
1	ZnO	Peanut (<i>Arachis hypogaea</i>)	Improved the growth and yield of peanut ¹²⁶
		Cluster bean (<i>Cyamopsis tetragonoloba</i> L.)	Enhanced the lengths of shoot and root, and contents of chlorophyll, total soluble leaf protein, rhizospheric microbial population, and P nutrient-mobilizing enzymes including phytase, acid, and alkaline phosphatase (foliar up 10 mg/kg) ¹²⁷
		Ryegrass (<i>Lolium perenne</i>)	Found responsible for retardation of roots and shoots of ryegrass ¹²⁸
		Cabbage (<i>Brassica oleracea</i> var. <i>capitata</i> L.) and maize (<i>Zea mays</i> L.)	Reported to be toxic to the early-stage development and growth of maize and cabbage. However, toxicity depends upon the dose of nanoparticles (NPs) and type of plant ¹²⁹
		Bacteria (<i>Bacillus subtilis</i> , <i>E. coli</i>)	Found more lethal for <i>B. subtilis</i> than <i>E. coli</i> . 10 ppm of ZnO reduced the growth of 90% <i>B. subtilis</i> , while even 1,000 ppm of ZnO arrested the growth of <i>E. coli</i> by 48% only ¹³⁰
		Bacteria (<i>Pseudomonas putida</i>)	Inhibition of bacterial growth significantly (even at 1 mg/L concentration) ¹³¹
		Bacteria (Rhizobiales, Bradyrhizobiaceae, Bradyrhizobium)	Arrested the growth of bacterial communities and reduced diversity (500 mg/kg soil for nano-ZnO) ¹³²
		Zucchini (<i>Cucurbita pepo</i>)	Reduced and delayed the seed germination process and also reduced the biomass of zucchini significantly ¹³³
		Mouse-ear cress (<i>Arabidopsis thaliana</i>)	Found to be very toxic to this plant, arrested the germination of the plant in 94% of seeds, and completely halted the root elongation process even at 400 mg/L NPs concentration ¹³⁴
		Soybean (<i>Glycine max</i>)	Root growth was impacted ¹³⁵
2	SiO ₂	Maize (<i>Z. mays</i> L.)	Enhanced plant biomass and reduced total organic compounds, such as phenols, aldehydes, and ketones ^{136,137}
		Tomato (<i>Lycopersicon esculentum</i> Mill)	Improved seed germination, seedling vigor index with respect to the control group (up to 8 g/L) ¹³⁸

(Continued)

TABLE 14.2 (Continued)

Effect of Different Nanoparticles on Germination and Growth of Plants or Microbes

Sl. No.	Nanoparticles	Crop/Plant/Microorganism	Effect on Crop/Plant/Microorganism
3	Al ₂ O ₃	Mouse-ear cress (<i>A. thaliana</i>)	No impact on root length and leaves count till 400 mg/L, but root length and leaves count decreased significantly at 2,000 and 4,000 mg/L concentrations ¹³⁴
		Lupin (<i>Lupinus</i> sp.) and wheat (<i>Triticum</i> spp.)	No signs of toxicity were observed up to the concentration of 2,000 mg/L ¹³⁹
		Bacteria (<i>B. subtilis</i> , <i>E. coli</i>)	Mild toxicity due to reactive oxygen species (ROS) production ¹³⁰
		Maize (<i>Z. mays</i>), cucumber (<i>Cucumis sativus</i>), soybean (<i>G. max</i>), wild cabbage (<i>B. oleracea</i>), carrot (<i>Daucus carota</i>)	Showed phytotoxicity to these plants; however, loading of phenanthrene significantly reduced the toxicity ¹⁴⁰
		Cucumber (<i>C. sativus</i>)	No impact on germination and root elongation of this plant ¹⁴¹
4	MWCNT	Mouse-ear cress (<i>A. thaliana</i>)	No impact on root elongation and leaves count till 4,000 mg/L concentration ¹³⁴
		Rape (<i>Brassica napus</i>), radish (<i>Raphanus sativus</i>)	No effect observed on seed germination and root elongation ¹⁴¹
		Zucchini (<i>C. pepo</i>)	Reduced and delayed the germination process and resulted in loss of biomass of zucchini ¹³³
5	TiO ₂	Rice cell (<i>Oryza sativa</i> L.)	Accumulation of MWCNTs in rice cell led to decrease in cell viability due to increase in ROS ¹⁴²
		Wheat (<i>Triticum aestivum</i>)	No effect on growth of root, shoot and leaf observed ¹⁴³
		Spinach (<i>Spinacia oleracea</i>)	Nano-anatase TiO ₂ increased the fresh weight, dry weight, and contents of total nitrogen, NH ₄ ⁺ , oxygen, chlorophyll, and protein of spinach; however, condition of spinach did not improve under N-deficient condition ¹⁴⁴
		Wheat (<i>T. aestivum</i>)	No effect on growth of root, shoot and leaf observed ¹⁴³
		Willow tree	No adverse effect observed on the willow trees ¹⁴⁵

(Continued)

TABLE 14.2 (Continued)
Effect of Different Nanoparticles on Germination and Growth of Plants or Microbes

Sl. No.	Nanoparticles	Crop/Plant/Microorganism	Effect on Crop/Plant/Microorganism
		Maize (<i>Z. mays</i> L.)	The presence of NPs was found to reduce the hydraulic conductivity of excised roots due to clogging of roots; however, its effect on long-term shoot growth was not observed ^{146,147}
		Bacteria (Rhizobiales, Bradyrhizobiaceae, Bradyrhizobium)	Reduced soil-specific bacteria and reduce their diversity ¹³²
		Bacteria (<i>B. subtilis</i> , <i>E. coli</i>)	These NPs are found to be more lethal for <i>B. subtilis</i> than <i>E. coli</i> . 5,000 ppm of TiO ₂ inhibits 72% of <i>E. coli</i> , whereas 2,000 ppm inhibit 99% of <i>B. subtilis</i> ¹³⁰
6	SWCNTs	Cabbage (<i>B. oleracea</i>), carrot (<i>D. carota</i>), cucumber (<i>C. sativus</i>), onion (<i>Allium cepa</i>), tomato (<i>Lycopersicon esculentum</i>), lettuce (<i>Lactuca sativa</i>)	Exposure to CNTs facilitated the root elongation of cucumber and onion, whereas root elongation was negatively affected for lettuce and tomato. No effect was observed on the root elongation of cabbage and carrot ¹⁴⁷
		Rice plant (<i>O. sativa</i>)	At high concentration, uptake of water and nutrients by the plant was impeded due to blockage of the plant roots and root hairs by surface-adsorbed nanotubes ¹⁴⁸
7	Fe ₃ O ₄	<i>Cucurbita maxima</i> (pumpkin) Lettuce (<i>L. sativa</i>), cucumber (<i>C. sativus</i>)	Significant amount of Fe ₃ O ₄ was found in the roots and leaves of pumpkin plant ¹⁴⁹ The presence of Fe ₃ O ₄ NPs showed significant reduction in germination index and root elongation of both the plants ¹⁵⁰
		Mouse-ear cress (<i>A. thaliana</i>) Ryegrass (<i>L. perenne</i> L.), pumpkin (<i>Cucurbita mixta</i>)	Root elongation was affected at concentration ranging from 400 to 4,000 mg/L ¹³⁴ Caused more oxidative stress and induced higher antioxidant enzyme for both the plants ¹⁵¹
8	CeO ₂	Rape (<i>B. napus</i>), radish (<i>R. sativus</i>), wheat (<i>T. aestivum</i>), lettuce (<i>L. sativa</i>), cabbage (<i>B. oleracea</i>), tomato (<i>L. esculentum</i>), cucumber (<i>C. sativus</i>)	Root length of lettuce was reduced by 33.6%, but no effect on other plants ¹⁵²

(Continued)

TABLE 14.2 (Continued)
Effect of Different Nanoparticles on Germination and Growth of Plants or Microbes

Sl. No.	Nanoparticles	Crop/Plant/Microorganism	Effect on Crop/Plant/Microorganism
		Wheat (<i>T. aestivum</i>) Soybean (<i>G. max</i>)	No effect on growth of root, shoot and leaf observed ¹⁴³ Seed germination was adversely impacted at a concentration of 2,000 mg/L and above. ¹³⁵
9	Yb ₂ O ₃	Rape (<i>B. napus</i>), radish (<i>R. sativus</i>), wheat (<i>T. aestivum</i>), lettuce (<i>L. sativa</i>), cabbage (<i>B. oleracea</i>), tomato (<i>L. esculentum</i>), cucumber (<i>C. sativus</i>)	Root elongation of all six plants was adversely affected. Among all these plants, wheat was least affected ¹⁵²
10	Gd ₂ O ₃	Rape (<i>B. napus</i>), radish (<i>R. sativus</i>), wheat (<i>T. aestivum</i>), lettuce (<i>L. sativa</i>), cabbage (<i>B. oleracea</i>), tomato (<i>L. esculentum</i>), cucumber (<i>C. sativus</i>)	Root elongation of all six plants was adversely affected. Among all these plants, wheat was least affected ¹⁵²
11	La ₂ O ₃	Rape (<i>B. napus</i>), radish (<i>R. sativus</i>), wheat (<i>T. aestivum</i>), lettuce (<i>L. sativa</i>), cabbage (<i>B. oleracea</i>), tomato (<i>L. esculentum</i>), cucumber (<i>C. sativus</i>)	Root elongation of all six plants was adversely affected. Among all these plants, wheat was least affected ¹⁵²
12	Pd	Kiwi <i>Hordeum vulgare</i> L. cv. Barke (barely) Lettuce (<i>L. sativa</i>)	Pd NPs changed the pollen morphology of kiwi fruit and got accumulated in the grains more rapidly and to a greater extent than soluble Pd(II) ¹⁵³ Pd particles caused stress in the leaves of the plants which led to decrease in the length of leaves ¹⁵⁴ Pd NPs at low concentration (P value of 0.002) helped in plant growth after 15 days of incubation ¹⁵⁵

(Continued)

TABLE 14.2 (Continued)

Effect of Different Nanoparticles on Germination and Growth of Plants or Microbes

Sl. No.	Nanoparticles	Crop/Plant/Microorganism	Effect on Crop/Plant/Microorganism
13	Ag	Onion (<i>A. cepa</i>)	Ag NPs demonstrated dose-dependent genotoxicity on the root tip cells of onion. Increase in the dose of NPs led to decrease in the mitotic index of cells and increase in chromosomal aberrations including breakdown of chromosomes and even cell wall disintegration ¹⁵⁶
		Lettuce (<i>L. sativa</i>), cucumber (<i>C. sativus</i>)	Ag NPs negatively affected the germination index and root elongation of both the plants ¹⁵⁰
		Cabbage (<i>B. oleracea</i> var. <i>capitata</i> L.) and maize (<i>Z. mays</i> L.)	Dose-dependent adverse effects of Ag NPs were observed in the early-stage growth of both the plants. However, toxicity of Ag ionic salt was more than Ag nanoparticles ¹²⁹
		Zucchini (<i>C. pepo</i>)	Reduced and delayed seed germination process and resulted in loss of biomass of zucchini ¹³³
15	Cu	Mung bean (<i>Phaseolus radiates</i>), wheat (<i>T. aestivum</i>)	Cu NPs were found to inhibit the growth of both the plants and accumulate in the plants in dose-dependent manner ¹⁵⁷
		Zucchini (<i>C. pepo</i>)	Reduced and delayed seed germination process, reduced root length significantly, and ascribed to loss of zucchini biomass ¹³³
		Lettuce (<i>L. sativa</i>)	Cu NPs at higher concentration (P value of 0.003) and a combination of Au and Cu NPs (P value of 0.006) significantly influence the plant growth after 15 days of incubation ¹⁵⁵

(Continued)

TABLE 14.2 (Continued)
Effect of Different Nanoparticles on Germination and Growth of Plants or Microbes

Sl. No.	Nanoparticles	Crop/Plant/Microorganism	Effect on Crop/Plant/Microorganism
16	Al	Rape (<i>B. napus</i>), radish (<i>R. sativus</i>), ryegrass (<i>L. perenne</i>), lettuce (<i>L. sativa</i>), corn (<i>Z. mays</i>), cucumber (<i>C. sativus</i>)	Observed no effect of seed germination process but enhanced roots of radish and rape, whereas reduced the roots of ryegrass, lettuce, and corn ¹⁴¹
17	Zn	California red kidney bean (<i>Phaseolus vulgaris</i>), ryegrass (<i>L. perenne</i>) <i>B. napus</i> (rape), <i>R. sativus</i> (radish), <i>L. perenne</i> (ryegrass), <i>L. sativa</i> (lettuce), <i>Z. mays</i> (corn), <i>C. sativus</i> (cucumber)	These particles did not have any adverse effect on growth of both the plants. But, particles found accumulated in the leaves of ryegrass ¹⁵⁸ Zn NPs were found to be inhibiting the germination process of <i>L. perenne</i> (ryegrass), and they affected the root elongation of all these plants ^{128,141}
18	Au	Lettuce (<i>L. sativa</i>) Lettuce (<i>L. sativa</i>), cucumber (<i>C. sativus</i>)	Au NPs at low concentration (P value of 0.008) and combination of Au and Cu NPs helped in growth and increased shoot/root ratio in 15 days ¹⁵⁵ No adverse effect on seed germination and root elongation was observed for both the plants ¹⁵⁰
19	Si	Zucchini (<i>C. pepo</i>) Lettuce (<i>L. sativa</i>)	Arrested the seed germination process completely ¹³³ Si NPs at higher concentration (P value of 0.0016) helped in plant growth and increased the shoot/root ration after 15 days of incubation ¹⁵⁵

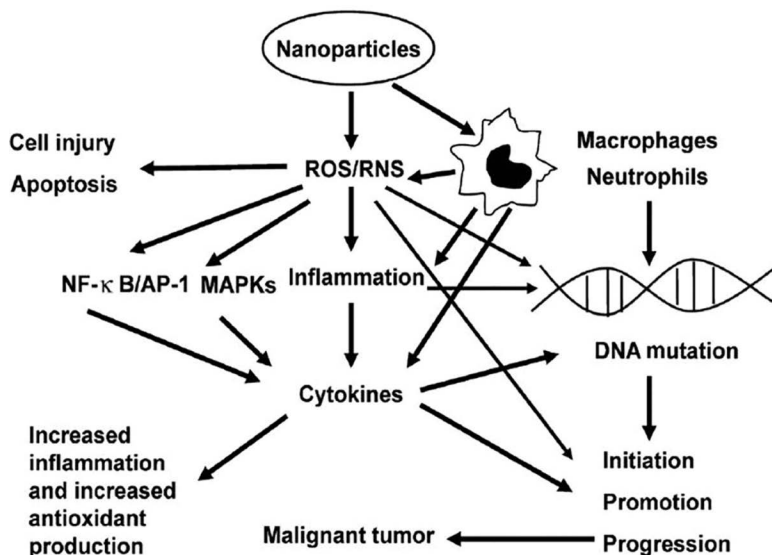


FIGURE 14.5 Potential mechanistic linking of nanoparticles exposure to toxic and genotoxic effects. Exposure to NPs may lead to oxidative stress due to increased production of ROS and downstream signaling responses that promote persistent inflammation and produce toxic and genotoxic effects. NPs, nanoparticles; ROS/RNS, reactive oxygen species/reactive nitrogen species.¹⁷¹

of events and inflammation are reported to activate the mitogen-activating protein kinase (MAPK), redox-sensitive transcription factors, nuclear factor kappa B (NF- κ B), and activating protein 1 (AP-1) which events play important roles in gene expression.^{166,167,178} NPs are also reported to activate the redox-responsive NF- κ B and AP-1 transcription factors in cells.^{164,180–182}

14.4 CONCLUSIONS AND FUTURE DIRECTIONS

With the advancement of science and technology, more and more amount and varieties of nanomaterials have been produced. Apart from these, natural events are also contributing to generation of a large amount of nanomaterials on each passing day. Hence, all living beings including human are getting exposed to an increased amount of NPs. The absence of proper regulations/guidelines in handling and disposal of nanomaterials may adversely affect the environment and all living organisms. Current research is directed toward evaluation of NPs toxicity in living organisms; however, research is very limited due to the increasing number and varieties of NPs. Long-term toxicity of these NPs and their potential environmental impacts need to be assessed. All the stakeholders including the industries, government, and society should take the responsibility to ensure safe handling and disposal of these NPs for a better world tomorrow.

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15 The Role of Nanotechnology in the Management of Water Toxicity

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15.1 INTRODUCTION

Untreated domestic and industrial wastewater contains harmful pathogens and hazardous substances (Bai et al. 2013). Wastewater from a variety of sources poses a health risk, and wastewater treatment plays a critical role in disease prevention across the globe (Akpore and Muchie 2011, Cairncross and Feachem 2018). Recycling and reuse of wastewater in an affordable and effective way is necessary for pollution control and disease control around the world (Piadeh et al. 2014, Salgot and Folch 2018). Conventional wastewater treatments, which are classified as preliminary, primary, secondary, and tertiary treatments, involve traditional methods such as grit chambers, primary sedimentation tanks, anaerobic digestion, trickling filters, and rotating biological contactors. Physicochemical treatment, combined biological-physical treatments, physical membrane-based separations, precipitation, distillation, reverse osmosis, electrodialysis, ion exchange, and freeze desalination have also been used for water treatment. The conventional wastewater treatment methods have several limitations such as partial pollutant removal, generation of toxic metabolites in the sludge, and high energy requirement

(Sonune and Ghate 2004). Applications of nanomaterials have been proven to have great efficiency in several fields like electronics, information technology, medical, health care, energy, food safety, and environment. Nanotechnology has already made a major advance in water purification and wastewater treatment. Nanomaterials are finding the applications in affordable detection and treatment of pollutants in water (Santhosh et al. 2016, Madhura et al. 2019). Nanomaterials eliminate contaminants from wastewater by different approaches such as photocatalysis, adsorption, filtration, antimicrobial property, and affinity towards specific substances through molecular imprinted polymers (Figure 15.1). The nanomaterial is a material that is deliberately produced in the size range from 1 to 100 nm to have specific characteristics. As per Kreyling et al. (2010), any particle is considered as a nanomaterial if it has a volume-specific surface area equal to, or greater than, $60 \text{ m}^2/\text{cm}^3$. Furthermore, as per the definition, the term “nano” is also applicable if any fraction of a material is in nanoscale.

This review highlights the benefits of nanotechnology in wastewater treatment after modifying the materials to nanoscale to obtain superior characteristics than macromolecule. Photocatalysis, filtration, adsorption, antimicrobial treatments, and affinity-based capturing of target molecules are well-established methods, and applications of nanomaterials in these methods have gained momentum for wastewater treatment. Different types of nanomaterials that are capable of removing contaminants from wastewater are summarized in Table 15.1. These methods have been considerably improved by the use of nanomaterials and revolutionized wastewater treatment methods. Different types of nanomaterial applications in wastewater treatment are discussed in this review.

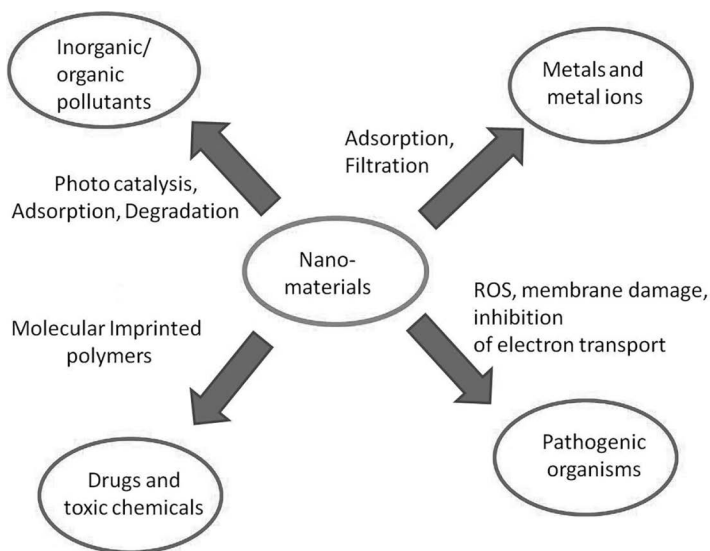


FIGURE 15.1 Various approaches of nanomaterials for the removal of contaminants from wastewater.

TABLE 15.1
Summary of Various Types of Nanomaterials for Wastewater Treatment

S.No.	Type of Nanomaterials	Examples	References
1	Nanocatalysts	TiO ₂ , zero-valent metallic, bimetallic nanoscale particles	Byrne et al. (2002), Kwon et al. (2008)
2	Nanosorbents	Carbon aerogels, graphene, or carbon nanotube-coated sponges, graphene foams or sponges, carbon coatings, activated carbon, porous carbon nanoparticles, and carbon fibers	Mauter and Elimelech (2008)
3	Nanostructured membranes	Inorganic-based nanosorbents	Ray et al. (2015)
		Polymer-based nanosorbents	Pan et al. (2009), Zhao et al. (2011)
		Inorganic nanostructured membranes	Li and Lee (2009), Kazemimoghadam (2010)
		Carbon-based nanomembranes	Lou et al. (2016)
4	Antimicrobial nanomaterials	Hybrid nanostructured membranes	Lind et al. (2009)
		Bionanomembranes	Kaufman et al. (2010)
		Inorganic/antimicrobial nanomaterials	Li et al. (2008), Raghupathi et al. (2011)
		Carbon-based antimicrobial nanomaterials	Deguchi et al. (2001), Brady-Estévez (2008)
		Natural antimicrobial nanomaterials	Gazit (2007)
5	Molecularly imprinted polymers (MIPs)	Peptides, chitosan MIPs with antibodies and biological receptors	Huang et al. (2015), Xue et al. (2013)

15.2 PHOTOCATALYSIS BY NANOCATALYSTS

Photocatalysis is employed to accelerating the oxidation and reduction reactions on the surface of a photocatalyst substance which is activated in the presence of UV-VIS light radiation (Fox and Dulay 1993). The mechanism of photocatalysis is summarized in Figure 15.2. The absorption of light by the photocatalyst substance produces valence band such as holes and conduction band such as electrons. Positive holes in the valence band oxidize the organic pollutants in wastewater and produce CO_2 , water, and degradation products. A positive hole also reacts with water and produces hydroxyl radicals which also oxidize organic pollutants. Electrons in the conductive band react with oxygen and produces anion radical superoxides which can convert the organic pollutants in wastewater and produce CO_2 , water, and degradation products (Mills and Le Hunte 1997, Dalrymple et al. 2010). Photocatalysis has been employed in water treatment plants for over two decades (Herrmann et al. 1993). Grafting polyethylenimine (PEI) on carbon nitride (C_3N_4) nanosheets exhibited enhanced photocatalytic bactericidal activity by electrostatic adhesion (Zeng et al. 2020). Polyvinyl chloride/ TiO_2 nanotubes, fabricated by a simple annealing process, significantly improved photocatalytic performance under visible light (Phat et al. 2020).

Several photocatalysts such as TiO_2 , zinc oxide, iron oxide, gadolinium oxide, and antimony oxide are being used in wastewater treatment. TiO_2 is the most effective photocatalyst and has been extensively used as an environmental remediation catalyst. It has gained importance for use in water and wastewater

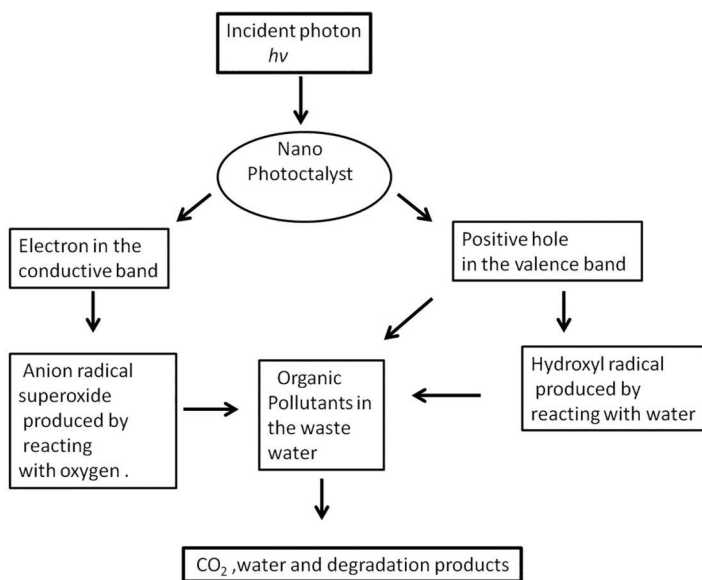


FIGURE 15.2 Schematic diagram of the photolytic mechanism of nanophotocatalysts for the degradation of pollutants.

treatment studies because it exhibits high photocatalytic activity. It is preferred over other photocatalysts due to its high thermal stability, dielectric properties, high resistance to chemical corrossions, non-toxicity, and cost effectiveness (Lazar et al. 2012). Nanosized TiO_2 particles are more reactive than larger particles due to their larger surface area (Byrne et al. 2002, Kwon et al. 2008). Organic pollutants such as methyl orange, rhodamine B and methylene blue, p-nitrophenol, eriochrome black T, reactive red 198, cephalixin, and Congo red can be reduced by photocatalysis methods (Reddy et al. 2016). Nanophotocatalysts can be synthesized by photo sonochemical, precipitation, emulsion solvent, evaporation method, grafting, solution processing method, and one-pot solvothermal methods (Fadlalla et al. 2019).

Significant research has been done in the field of photocatalysis for the wastewater treatment, and application of nanomaterials as photocatalysts has gained momentum recently due to their tunable surface and electronic properties. Despite promising advantages, nanophotocatalysts have to overcome with some limitations such as wide band gap and initiation of photocatalytic activity only to UV light. The use of visible light is a challenge in the photocatalytic activity of nanophotocatalysis, and it needs significant progress in the research. Modification of photocatalytic activity can be done by use of doping agents such as Cr, Si, Co, Mg, Mn, Fe, Fe, Al, In, and Ga.

15.3 NANOFILTRATION

Nanofiltration is done by a charged membrane with a pore size of 1–5 nm which can be operated at low pressure. Use of nanofiltration has gained momentum in water treatment due its combined properties, ultrafiltration, and reverse osmosis. Lower energy consumption and higher rejection are other important parameters to consider the nanofiltration for wastewater treatment (Mohammad et al. 2015). There are majorly four types of membranes being used for the wastewater treatment. Microfiltration contains a porous isotropic membrane with a pore size of 50 nm to 1 μm , and the transfer of the particles through this membrane is governed by Darcy's law and works on sieving and adsorptive mechanisms. This method is suitable for solutions with solid particles. Ultrafiltration contains a porous asymmetric membrane with a pore size of 5–20 nm, and the transfer of the particles through this membrane is governed by Darcy's law and works on sieving and preferential adsorption mechanisms. This method is suitable for solutions with colloids and/or macromolecules. Reverse osmosis membranes consists of a nonporous membrane, and the transfer of the particles is governed by Fick's law and solutes migrate by diffusive mechanisms. This method is suitable for solutions with solid particles (Bunani et al. 2013). Separation of rare earth elements and iron from waste permanent magnets (WPMs) leach liquor was studied using solvent extraction followed by a hollow fiber-supported liquid membrane. This study shows promising results in the lab scale for the recycling of rare earth elements and iron from industrial waste materials (Ni'am et al. 2020). In a recent study, Liang et al. (2020b) demonstrated the synthesis of polyamide

membranes for surfactant-assembly regulated interfacial polymerization method for separation of sub-1 Å ions and small solutes.

Nanostructured membranes like carbon nanotubes can filter bacteria and viruses. Alumina membrane formed from A-alumoxane can filter synthetic dyes. Alumina membranes functionalized with poly(styrene sulfonate) or poly(allylamine hydrochloride) can filter divalent cations. Metal ions can be filtered by silica- and cellulose-based membranes functionalized with amino acid homopolymers. Trichloroethylene can be filtered by nanostructured materials such as Pt/Fe-laden cellulose acetate film and zero-valent Fe-laden cellulose acetate. Membrane, Ni/Fe- or Pd/Fe-laden polyacrylic acid/polyether sulfone composite membranes, Ni/Fe laden cellulose acetate membrane. Alumina or polymeric membranes with gold nanoparticles can filter 4-nitrophenol. Polymer-impregnated ceramic TiO₂ filters can filter polycyclic aromatic hydrocarbons. Polymer-impregnated ceramic alumina and silicon-carbon filters can filter trihalogen methanes and pesticides (Theron et al. 2008).

An integrated process of nanofiltration and evaporation in rubber wastewater treatment results in a reduction of total hardness concentrate. The permeate obtained by this process could be reused as boiler feed water (Xin et al. 2013). Membrane bioreactor (MBR) and nanofiltration in dairy industry had rejected 99% of COD and 93.1% of total solids, and the permeate could be reused for boiler and cooling water (Andrade et al. 2015). A combination of biological (full-scale plant) processes with MBR and nanofiltration-reverse osmosis integrated system resulted in the removal of COD, hardness, conductivity, fluoride, and chloride ion from coking wastewater treatment, which meets the standards for industrial reuse (Jin et al. 2013). Nanofiltration by the secondary effluent of textile wastewater treatments resulted in obtaining purified water for textile industry reuse. The best rejection of wastewater was showed by a combination of ultrafiltration with NF90 and reverse osmosis. This water can be reused for steam generation, washing factories, and vehicles (Racar et al. 2017).

Even though soluble elements cannot be separated from the water by using nanofiltration, valence ions and organic elements can be removed. As nanofiltration does not involve any chemicals, there will be no threat to the environment. Pretreatment of water is required before the nanofiltration, and further treatment of concentrate is one of the major challenges in nanofiltration. Cost and life time of the membranes and membrane fouling are the major drawbacks for nanofiltration.

15.4 NANOSORBENTS

Nanosorbents for water treatment are defined as nanoparticles ranging from 1 to 100 nm size which have large surface area with high reactivity and are highly effective in isolating a wide variety of pollutants. Regeneration by desorption of nanosorbents and enhancement of surface reactivity of nanosorbents are the important characteristics of new-generation nanosorbents (Sharma et al. 2009, Sadegh et al. 2017). In a recent study, guar gum/activated carbon nanocomposite was synthesized and utilized for the removal of Congo red dye from

wastewater. By optimization of physicochemical parameters, kinetic and thermodynamic studies revealed that guar gum/activated carbon nanocomposite can be used as an effective and efficient adsorbent (Gupta et al. 2020). Alginate/pectin nanocomposite was synthesized for the removal of crystal violet dye from aqueous solution. This porous and amorphous nanocomposite exhibited spontaneous nature of adsorption and exceptional regeneration properties (Mirza and Ahmad 2020). Poly(methyl methacrylate)-grafted alginate@Cys-bentonite copolymer hybrid nanocomposite was synthesized by in situ chemical oxidative polymerization for the removal of heavy metals by electrostatic adsorption-coupled reduction. The adsorption process was found to be spontaneous and endothermic in nature (Hasan and Ahmad 2019). Xanthan gum/montmorillonite nanocomposite can be utilized as an adsorbent for the removal of heavy metals such as Pb(II) from wastewater (Mirza and Ahmad 2018). Chitosan-iron oxide ($\text{CS-Fe}_2\text{O}_3$) nanocomposite and guar gum/bentonite nanocomposite were synthesized and studied for the removal of toxic elements from wastewater, and these nanocomposites found to be excellent adsorbents for the removal of contaminants from wastewater (Ahmad and Mirza 2018a, 2018b). In a study, sulfidated nanoscale zerovalent iron exhibited complete degradation of tetra bromobisphenol A in the dynamic two-step anoxic/oxic process (Wu et al. 2019).

Mesoporous magnetic $\gamma\text{-Fe}_2\text{O}_3$ goethite nanoparticles, ascorbic acid-coated Fe_3O_4 nanoparticles, magnesium ferrite nanocrystallites, TiO_2 nanoparticles, iron-doped titania nanoparticles coated on glass beads, cerium oxide nanoparticles, magnesium oxide nanoflakes, CuO nanoparticle, zirconium oxide nanoparticles, multiwall carbon nanotube–zirconia, and nanohybrid multiwalled boron nitride nanotubes are being used for the removal of arsenic (As(III) & As(V)) ions (Lata et al. 2016). Magnetic mesoporous silica modified with poly(1-vinylimidazole) oligomer, magnetic mesoporous silica, nanoparticles functionalized with chitosan–glutaraldehyde, poly(aniline-co-5-sulfo-2 anisidine) nanoparticles, magnetic nanoadsorbent, multiwalled carbon nanotubes, and poly(1-amino-5-chloroanthraquinone) nanofibrils are being used for the removal of mercury (Hg(II)) ions (Sharma et al. 2009). Mesoporous silica/multiwalled carbon nanotubes, magnetite nanoparticles, magnetic silica nanoparticles, chitosan-coated magnetic nanoparticles, ZnO nanoplates, magnesium oxide nanoparticles, zinc oxide nanoparticles, and chitosan/multiwalled carbon nanotubes composite are being used for the removal of copper (Cu(II)) ions. Ag/Fe bimetallic nanoparticles, nanosize zero-valent iron, nano-zirconiumvanadate particles, silver biological nanoparticles, and carbon nanotubes are being used for the removal of cobalt (Co(II)) ions (Ray and Shipley 2015). Smart magnetic graphene, ceria hollow, nanospheres, chitosan/clay nanocomposites, polypyrrole polyaniline nanofibers, multiwalled carbon nanotubes, polyglycidyl methacrylate graft with functionalized iron oxide mesoporous silica nanosorbent, and nano-alumina are being used for the removal of chromium (Cr(VI) and Cr(III)) ions (Sharma et al. 2009). Iron oxide nanoparticles, magnetic alginate nanobeads, nanoporous alginate-SBA-15 manganese oxide nanoparticles, methyl cellulose and nano-chitosan kaolin clay and nano-chitosan, nano-alumina graphene

oxide integrated with magnetic chitosan nanoparticles, mesoporous nano-silica particles, chitosan–montmorillonite nanocomposites, and polyacrylicacid/acrylonitrile–attapulgitite nanoparticles are being used for the removal of lead (Pb(II)) ions (Ray and Shipley 2015). Magnetic nanoparticles, nano-alumina carbonaceous nanosorbents, nano-silica multi-walled carbon nanotubes, cerium oxide, iron oxide, and titanium oxide are being used for the removal of cadmium (Cd(II)) ions (Thekkudan et al. 2016, Dinesha et al. 2017). Reuse and recycling is one of the major problems of nanosorbents, and research needs to be carried out to develop efficient methods for desorption of the used nanosorbents. Lack of greener methods for the synthesis of nanosorbents is also a major challenge for the usage of nanosorbents. Synthesis of nanosorbents by chemical methods may lead to the toxicity of the treated water.

15.5 ANTIMICROBIAL NANOMATERIAL TREATMENT

In wastewater treatment, formation of harmful disinfection byproducts is the major problem for conventional disinfectants such as free chlorine, chloramines, and ozone (Rice 1996, Kim et al. 1997). Use of antimicrobial nanomaterials in the wastewater treatment offers broad antimicrobial activity without generating any harmful byproducts. Moreover, these nanomaterials are inexpensive and highly soluble in water. Nanosized metals and metal oxides, carbon nanomaterials, and naturally occurring substances are being used for wastewater treatment (Li et al. 2008). Nanomaterials can exhibit antimicrobial activity by damaging the cell membrane, inhibiting the electron transfer, oxidizing the cell components, and producing secondary products like reactive oxygen species and metal ions (Hossain et al. 2014). Silver nanoparticles are well-established antimicrobial agents, and the mechanism of cell death involves release of Ag^+ ions, disruption of cell membrane and electron transport, and DNA damage. Application of silver nanoparticles includes the wastewater treatment and portable water filters (Sheng and Liu 2011). TiO_2 nanoparticles exhibit antimicrobial property by production of ROS and damage of cell membrane and wall. Applications of these nanoparticles include the organic contaminant degradation in water treatment systems. ZnO nanoparticles act by intracellular accumulation of nanoparticles and exhibit antimicrobial property through cell membrane damage, H_2O_2 production, and release of Zn^{2+} ions (Ma et al. 2014, Raghupathi et al. 2011). Chitosan nanoparticles can be used as flocculants in water and wastewater treatment, and these nanoparticles show antimicrobial effect by membrane damage and chelation of trace metals (Olivera et al. 2016). Fullerenes (nC60) show the antimicrobial effect that could be due to the ROS production and direct oxidation of cells (Wang et al. 2012).

Fate and behavior of nanomaterials in wastewater treatments needs to be studied to further evaluate the risk to the environment. Scale-up is still challenging, and the high concentration of antibacterial nanomaterials in treated water may be harmful to human health. Aggregation of small nanomaterials also needs to be addressed before it is used for wastewater treatment.

15.6 MOLECULARLY IMPRINTED POLYMERS (MIPs)

Molecularly imprinted polymers (MIPs) are imprinted polymers that contain a specific binding molecule and cavity for target molecules (Cormack et al. 2004). Binding of the target molecule is reversible, and it will help in the reuse of MIPs in the wastewater treatment for the removal of pollutants. High selectivity, mechanical strength, and resistance against a wide variety of chemicals are some of the important characteristics of MIPs, which make it attractive for the removal of pollutants from wastewater (Huang et al. 2015). The template molecule and the functional monomers were allowed to react to form a template-functional monomer complex. Subsequently, the cross-linker and the radical initiator were added to initiate the polymerization of the monomers. The resulting polymer with a template molecule was washed with suitable solvents to obtain MIPs which can be used for the removal of pollutants from wastewater (Figure 15.3). In a study, MIPs were prepared via precipitation polymerization and these MIPs exhibited the highest molecular recognition toward sinapic acid relative to non-imprinted polymers (Til et al. 2020). In a recent study, 4-nitrophenol was extracted from solid-phase wastewater by magnetic surface molecular-imprinted polymer synthesized by a microwave-assisted method (Liang et al. 2020a). In a study, core-shell magnetic MIPs were synthesized by using $\text{Fe}_3\text{O}_4@\text{SiO}_2$ as magnetic core and extracted allocryptopine effectively from the waste of *Macleaya cordata* (Wang et al. 2020). Ag_2S molecular imprint polymer TiO_2 nanocomposite was synthesized by a sol-gel deposition method, and the photolytic degradation of ethyl p-hydroxybenzoate was observed in wastewater (Liu et al. 2020).

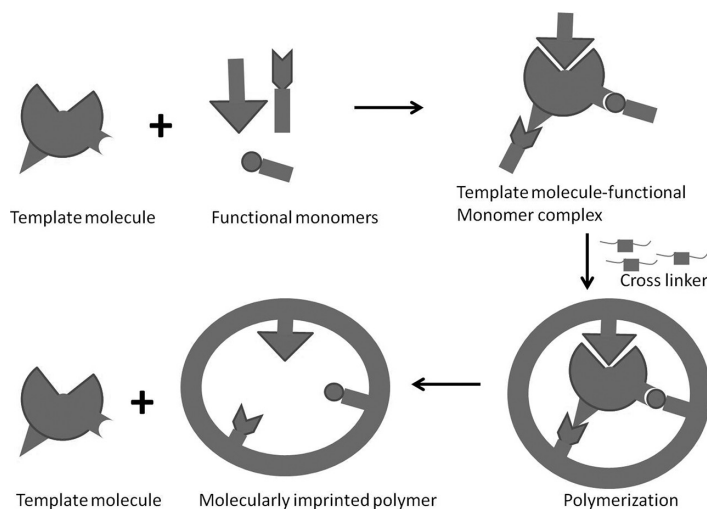


FIGURE 15.3 Schematic representation of the mechanism of molecular-imprinted polymers for the removal of target molecule.

MIPs composite with Fe_3O_4 can target analytes like estrogens, nitrophenol, sulfonamides, water-soluble acid dyes, and bisphenol A in wastewater treatment (Huang et al. 2015). MIPs composite with kaolinite and Fe_3O_4 can target bisphenol A in wastewater treatment (Guo et al. 2011). MIPs composite with chitosan Fe_3O_4 can target analytes like carbamazepine in wastewater treatment (Yu et al. 2011). MIPs composite with Au nanoparticles can target analytes like bisphenol A in wastewater treatment. MIPs composite with TiO_2 nanotubes can target analytes like estrogenic compounds in wastewater treatment (Xue et al. 2013). MIPs composite with carbon nanotubes can target analytes like triclosan estrone in wastewater treatment (Gao et al. 2010). MIPs composite with multiwalled carbon nanotubes can target analytes like 2,4-dichlorophenoxyacetic acid in wastewater treatment (Yang et al. 2013). MIPs composite with halloysite nanotubes can target analytes like 2,4,6-trichlorophenol in wastewater treatment (Pan et al. 2011). MIPs composite with carbon nanoparticles can target analytes like fluoroquinolone antibiotics in wastewater treatment (Tan et al. 2013). Application of MIPs in wastewater treatment plants is at its initial stage, and elucidation of fundamental mechanism plays a key role in designing MIPs for large-scale wastewater treatment plants. Synthesis of water-compatible MIPs and leakage of residual template from MIPs are the major challenges for the development of novel MIPs for wastewater treatment.

15.7 CONCLUSIONS

Nanotechnology has a great potential to find innovative solutions in wastewater treatment. Nanomaterials have several desired characteristics for wastewater treatment such as high surface to volume ratio, highly reactive and tunable surface, high adsorption, high affinity, and ability to form films by self-assembling on substrates. These properties of nanomaterials are useful in the removal of a wide variety of contaminants from domestic and industrial wastewater. High cost, release of nanomaterials to the environment, and health risks are the major drawbacks for the use of nanomaterials in wastewater treatment. These problems need to be studied for the safe and efficient use of nanomaterials in wastewater treatment. Retention and reuse of nanomaterials will be helpful in reducing the cost and controlling the environmental contamination. Green technologies in the fabrication of nanomaterials and long-term evaluation of ecotoxicity and health risks will enable nanomaterials as future candidates for wastewater treatment.

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16 Challenges in the Assessment of Nanotoxicity, Recommendations, and Safe-by-Design Nanomedicines to Counter Toxicological Problems

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16.1 INTRODUCTION

Nanotoxicology is a branch of science that deals with the study and application of nanomaterials (NMs) concerning their toxicity in humans and the environment. Nanotoxicology is emerging as an important sub-discipline of nanotechnology. Mainly nanotoxicological studies are proposed to govern the extent to which the

toxic properties threaten the environment and human beings [1]. The types of toxic exposure to nanoparticles (NPs) are as follows:

- a. Consumer exposure through the usage of NP-containing personal care products, cosmetics, and sunscreen preparations;
- b. Occupational exposure for workers in NM manufacturing and research;
- c. Environmental exposure through the increasing concentrations of NMs in groundwater and soil, which may produce a significant environmental risk [2].

Furthermore, the toxicity of NMs is roughly classified into two areas:

- a. Biological toxicity
- b. Environmental toxicity.

In biological systems, the nanostructured NPs enter the body via six principle routes, i.e., oral, intravenous, dermal, subcutaneous, inhalation, and intraperitoneal. In cellular systems, they can cause biological toxicity by DNA damage or ROS (reactive oxygen species) generation. This leads to tissue damage, inflammation, cytotoxicity, and organ failure from the deposition of NPs. And, it also causes fibrosis and allergies [3]. In environmental systems, non-degradable NPs are ready to deposit in the groundwater, leading to the production of environmental pollutants. Further, they may also be harmful to plants and microbes.

16.1.1 NANOTOXICOLOGICAL CLASSIFICATION SYSTEM

In nanotoxicological classification system (NCS), four classes (I–IV) of increasing hazards/toxicity are constructed on the concerns about size classes and biodegradability (see Figure 16.1). Low-hazard particles are linked to green, medium-hazard particles to yellow, and high-hazard particles to red traffic light. Consequently, it explains composite circumstances into vibrant information and enables the discrimination of NPs, in green, yellow, or red NPs.

Class I comprises the particles of no or the lowest risk, i.e. particles exceeding 100 nm to just inferior to 1,000 nm being biodegradable. Illustrations for this class of “green” NPs are nanoemulsions, liposomes, drug nanocrystals, and lipid NPs in the corresponding size range. These particles do not necessarily possess any risk earlier. Several of them can retain a slight risk in the case. They produce hazardous side effects throughout their presence, e.g. interaction with the immune system. In classes I and II, the persistency rises. Identical to class I NPs, the class II NPs are greater than 100 nm, but non-biodegradable. Class III NPs are smaller than 100 nm, but biodegradable. Classes II and III retain the moderate risk, i.e. yellow particles. NPs of class IV retain the maximum toxicity risks. They are smaller than 100 nm and can consequently access all cells; on top, they are not biodegradable. As a result, particles of this class are called “red” NPs.

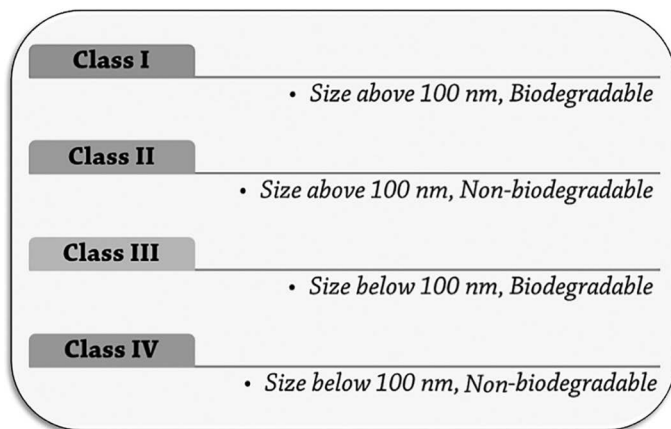


FIGURE 16.1 Nanotoxicological classification system (NCS) for an accurate distinction of nanotoxicological risks.

16.1.2 PHYSICOCHEMICAL PARAMETERS FOR TOXICITY ASSESSMENT

Cellular responses are openly associated with physicochemical parameters of NMs. There are numerous morphological factors on which nanotoxicological response depend such as size, surface morphology, charge, and composition.

16.1.2.1 Size of Nanomaterials

To resolve cytotoxic response, the size of NMs is a primary factor. It affects the internalization practice into cells and endocytosis process, which finally alters the intracellular chance of NMs. Most of the studies conclude that the smaller the size of NPs, the higher the degree of cytotoxicity [4,5]. In the case of quantum dots (QDs), the cytotoxicity and size depend upon the mode of preparation. QDs produced by using ligands like trioctyl phosphine lead to hydrophobic nature and more converted to hydrophilic, foremost to an increase in hydrodynamic radius of NMs [6].

16.1.2.2 Surface, Surface Coatings, and Charge

Cellular uptake mechanism and cytotoxicity rely on the morphology of NPs. NMs have different kinds of shapes, which includes spheres, needles, cubes, tubes, rods, etc. Membrane interactions during the internalization of NPs affect the nature of barriers. Researchers reported the formation of pores in cell membranes due to interactions of NPs, leading to an imbalance in an ionic concentration outside and inside of the cell [7]. Recently, Maysinger et al. [8] also studied the gold nanourchins whose surface morphology has an irregular shape. Surface coatings on NPs surface act as a connecting link between nano-cellular interfaces. Coating affects the interparticle interactions, cellular contacts, internalization, and cytotoxicity of materials [9]. Surface coating possesses separate charges which can

change the cytotoxicity of materials. Various studies show that positively charged NMs internalized more effectively and also result in more toxic effects than neutral or negatively charged particles [10,11]. Coatings roughly divided into three types based on interactions by Richards et al. [12]. These are covalent coatings having covalent bonding, the electrostatic surface coating having electrostatic interactions, and atomic layer deposition where chemical bond formed between molecules and coating material. Coating plays a crucial role in various NM applications in drug delivery, imaging, and cancer treatment [13,14]. The coating also shows significant effects when changed from single layer to double layer by varying hydrophobic and hydrophilic, respectively. It was observed that hydrophobic coating impart a high level of toxicity than the hydrophilic counterpart. NMs are internalized through lipid bilayer membrane structure where the charge on the membrane is negative. Hence, oppositely charged NPs pass through excellently due to electrostatic interactions, while negatively charged bound less efficiently.

16.2 CHALLENGES IN THE ASSESSMENT OF NANOTOXICITY

Due to the emerging research on the toxicity of NPs, many researchers addressed the practical issues and challenges to be taken into account in conducting toxicity studies with NPs:

- a. Physiochemical properties like size, shape, charge, composition, aggregation, etc. are essential to be characterized in detail at all the stages of nanotoxicity testing since these factors are playing major roles in interaction with biological systems.
- b. Maintaining exact dose concentration is often very difficult due to the factors like mass, dimension, surface area, structure, and aggregation.
- c. Selecting appropriate exposure route is likely to human exposures like oral, dermal, inhalation, and injection routes.
- d. Cellular uptake, trafficking, and exclusion play a key role in the toxicity of NPs. Hence, the size, shape, surface chemistry, and charge in the cells should be considered and the interaction of NPs with the target cells should also be taken into account.
- e. Interactions of NPs with biological components like protein, carbohydrate, and DNA may alter the biodistribution, immune response, metabolism, and physiochemical properties of NPs, which disturb the absorption, wrapping, and entanglement.
- f. Quantitative evaluations of NPs in the target organ and tissues are difficult since their lower particle size and less accumulation.
- g. The desired dose concentration is often much difficult to accomplish due to factors like NP engulfment by blood macrophages, accumulation of NPs in the reticuloendothelial system, aggregation due to the attachment of NPs to antibodies and complementary proteins, and NPs binding with blood vessel walls and blood clots.

16.2.1 IN ASSESSING THE TOXICITY OF NANOMATERIALS

With the accumulative custom and manufacture of NMs, occupational exposure is also growing. A further concern is associated with the environment and ecosystem disturbance. Some of these apprehensions have forced scientists to investigate and understand the potential adverse effects of engineered NMs on health and the environment and also explore the challenges to assess the toxicity of these materials. Still, it is a challenging task to investigate the exchanges of NPs with biological systems. One of the feasible reasons could be due to experimental methods and exact characterization involving toxicological assessment of NMs. However, human health is at considerable risk because of the toxicity of these nanotechnology-based products on exposure/intake through several routes (Figure 16.2) [15].

16.2.2 IN CHARACTERIZATION

To study the toxicity of any chemical substances, the characterization of materials plays a significant role. There are numerous techniques obtainable these days which can be used to characterize NMs in powder form, film, and in solution, and its interaction with biomolecules can be further studied (see Figure 16.3).

However, it becomes much more commanding and broad in the case of NMs due to the different shapes and sizes with variable surface area, charge and chemistry, crystallinity, porosity, agglomeration, solubility, etc. (see Figure 16.4). Further, the NMs produced from experiments must ensure their reproducibility and thus advanced trustworthiness of the results. Characterization of NMs requires highly sophisticated instruments and skilled human resources to study. The precise properties of NPs and their toxicity details are poorly understood. Thus, a more wide-ranging and comprehensive characterization, including size distribution, shape, surface area, surface chemistry, crystallinity, porosity, agglomeration state, surface charge, solubility, etc. is suggested for NMs to

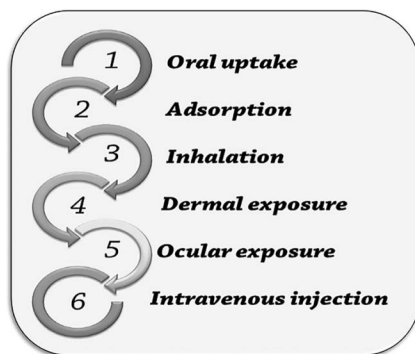


FIGURE 16.2 Exposure routes of NPs.

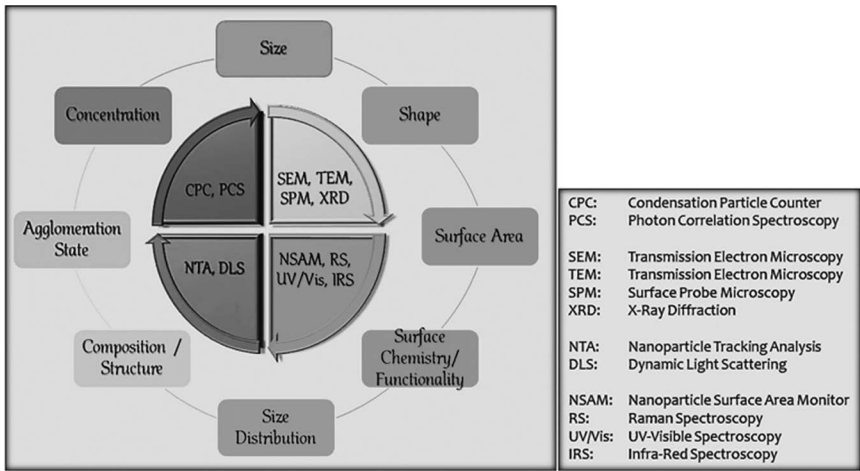


FIGURE 16.3 Characterization of NPs in different media.

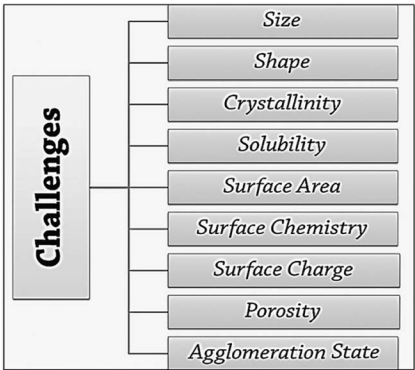


FIGURE 16.4 Challenges of characterization of NPs.

determine the perfect connection between their physicochemical properties and the biological effects they create [16]. Therefore, characterization techniques play a crucial role in the experimental findings of NMs.

16.2.3 NANOTOXICITY ASSAYS

To assess the extent of NP interference with toxicity assays and to determine if effects are predictable based on the physicochemical properties of the NP, we performed a systematic investigation of the accuracy of commonly used toxicity assays. At first, we have tested assay reliability only with NPs and assay components, and then several of the procedures were tested under more representative conditions. Assessments of NP toxicity need a set of design rules. During the earlier years,

in vitro toxic characterizations of NPs have been well summarized and compared. Marquis et al. [17] studied the analytical methods relating to proliferation, necrosis, apoptosis, DNA damage, and oxidative stress, which are associated with the main mechanisms of cytotoxicity. While *in vitro* studies have been performed widely [18], the significance of *in vivo* nanotoxicity studies is being highlighted [19]. Although many reviews have summarized the methods in both *in vitro* and *in vivo* studies in certain nanostructures in different model systems (Table 16.1), systemic evaluation remains indefinite. Fischer emphasized the importance of developing predictive models of NP toxicity assessment, whereas many researchers have focused on histological changes [20] and pharmacokinetic parameters like exposing [21], biodistribution [22,23], biochemistry metabolism, and clearance. However, exploration of nanotoxicity remains superficial in sacrificed animals.

TABLE 16.1
Summary of Five Basic Nanomaterial Properties and Their Potential Risks and Challenges

S. No.	Nanomaterial Properties	Description of the Risk
1	Size	• Reactivity and agglomeration of NPs is mostly size dependent • Well known that the agglomeration process is in slower rate in smaller particles
2	Impurity	• Intrinsically, NPs interact with impurities due to their high reactivity • Encapsulation becomes a prime necessity for solution-based NP synthesis (chemical route)
3	Agglomeration or aggregation	• Weakly bound (agglomeration) and fused particles are significant risk criteria as they lead to poor corrosion resistance, high solubility and phase change of NMs
4	Reactivity or charge	• NPs can be charged either by functionalization or spontaneous degradative reactions • Chemical species and their charge-related critical functional groups will be a significant factor for specific functionality and bioavailability of NMs
5	Contaminant dissociation	• The contamination of residual impurities in NPs is considered as a major risk factor • For example, sulfur impurities present in iron oxide NPs depending on the precursor used for their production (FeCl_3 or $\text{Fe}_2(\text{SO}_4)_3$) • Nickel, yttrium, or rubidium metal impurities may be present in carbon nanotubes (CNTs) that are adsorbed on the CNT surface
6	Recycling and disposal	• NMs are not bound to any hard-and-fast safe disposal policies

Based on the *in vivo* results of NP pharmacokinetics, comprising homeostasis regulation, systemically evaluating the impact of NPs on the foremost systems, including the hepatic, renal, digestive, pulmonary, hematological, cardiovascular, nervous, and immune systems, may provide profound insights into this field.

16.2.3.1 Systemic Assessment

Nanotoxicity can be verified from pathological changes, including morphologic changes and functional damage. The mechanisms underlying NPs' physiological interactions range from cells to organs and were discussed in the modern analysis [24]. Additionally, in terms of *in vivo* studies, the selection of animal models is the initial step. Although many animal models have been built, a standard and predictive model is still underdetermined. Besides the most common experimental mice and rats, zebrafish [25,26], rabbits [27], and *Caenorhabditis elegans* [28] were also used in nanotoxicology studies. An openly known genome and developed for application in toxicity investigation; mice and rats would be more appropriate models in nanotoxicology studies. *In vivo* assessments are allocated into two main categories. One includes tissue structure changes [29,30], apoptosis [31,32], and inflammation infiltration in the main organs. Consequently, combined with the exposing ways (ingestion, injection, transdermal delivery, and inhalation) and applications, appropriate assessment models could be established, especially for the drug delivery of NPs [33].

16.2.3.2 Biodistribution

Revealing the biodistribution of NPs in organs is beneficial to monitor the correction and adjustment of NPs. The most common way of exploring the distribution is to remove the main organs [27] or tissues (lung, liver, kidney, heart, spleen, pancreas, brain, fat, and muscle) after animal sacrifice, by using physical detection methods track the NPs according to their respective characteristics. For some metal NPs, their intrinsic properties could be probed by specific instruments. Certainly, gold composite nanodevices in mouse tumor tissues could be explored by instrumental neutron activation analysis [22]. Si and Cd [29] concentrations could be determined by inductively coupled plasma optical emission spectrometry. Some fluorescent-labeled particles [34] in tissue homogenates were analyzed by a plate reader. In some other materials [35], like QDs with heavy metal cores, it was more appropriate to apply multiplexing and multicolor imaging through single-particle Förster resonance energy transfer assays [36].

However, qualitative analysis with a light microscope (LM) and electron microscope (EM) failed to show sufficient and significant evidence in current studies. Other imaging techniques including typical imaging instruments like magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single-photon emission computed tomography, optical imaging, and ultrasound were introduced in the modern analyses [37,38]. Additionally, multi-scale, real-time, and quantitative technologies were reported

lately [39]. As some imaging contrast agents have potential unintentional toxicity, these tracing methods may aggravate the toxicity of NPs. Also, the combination of contrast agents may modify the features and subsequently change the biodistribution of NPs.

16.2.3.3 Gastrointestinal System

Compared with injection, oral administration of drugs or others is considered favorable due to convenience and compliance for patients. Drug bioavailability over spoken administration, however, is restricted because of physiological barriers of the gastrointestinal tract (GIT). With the development of nanocapsule transportation, certain new drug delivery systems are slowly being developed, e.g. polymeric NP [40], which prevents from inactivation and degradation by acid and enzymatic barriers of the GIT. There are a majority of researches that report the benefits of NPs, such as the reversal of nonsteroidal anti-inflammatory drug-induced gastrointestinal injury [41] and radioprotection [42] from cancer radiotherapy. Cerium oxide NPs [43] were also reported to protect the gastrointestinal epithelium from reactive oxygen loss.

16.2.3.4 Pulmonary System

As conservation anxieties, the initial exploration of pulmonary toxicity was absorbed on inhalable particles. NP, as a novel material with targeting and non-invasive structures in the drug delivery system, is an added group linked to pulmonary impact. To sign pulmonary accumulation of NPs, MRI [44] was recycled to see antibody-conjugated superparamagnetic iron oxide (SPIO) NPs in a lipopolysaccharide-induced enduring obstructive pulmonary disease mice model. The finest trade method is intratracheal instillation for long-standing and short-term [45] toxicity assessments, in which the bronchoalveolar lavage (BAL) fluid is collected for chemical analysis, with lactate dehydrogenase (LDH) assay.

16.2.3.5 Nervous System

In the nervous system, assessments were absorbed on the drug delivery of solid lipid nanoparticles (SLNs) in the brain. Mainly, brain therapy was enriched, and drug permeability across the blood–brain barrier was assessed with radiography techniques like PET and PET/CT system [46]. Magnetite-labeled NPs can turn as a gap mediator in rat brain MRI [47]. Confocal fluorescence training was also useful to evaluate the interest of brain endothelial cells. Temporarily, acute toxicity was imagined by brain histology analysis [48] and fluorescence imaging. An exterior electrical stimulus is one mutual method used in neurophysiology learning, in which caudal sensory nerve potentials [49] are noted centrally from the tail. Due to the high oxidative metabolism in the brain, the purpose of brain mitochondria, which is certainly isolated by differential centrifugation, is a good catalogue to calculate brain nerve injury. The respiratory chain of the mitochondria provides prospects for potential instrument study.

16.2.3.6 Immune and Reproductive System

The interfaces among all novel chemical and biological things of NPs and the immune system need to be illuminated previously to application in medicine and biology. Artificial variation of NPs concludes their immunogenicity, which may form basis for immunotoxicity by relating to the immune system [50]. Because of the difficulty of the immune system, the toxicity assessment of NPs depends on overall changes of immune cytokines and molecules, immune organs, hematological system, etc. For example, the generation and declaration of ILs and tumor necrosis issues are regularly examined upon the custom of NPs at high feature ratio, some metal or cationic. The toxicity assessment of the reproductive system is a relatively long-term study. Only limited researches have focused on this benevolent chronic study. Up to now, several *in vivo* and *in vitro* models have been established to study NP-associated toxicity in relative organs of this system. Unlike other assemblies, the reproductive system is sexually divided into female and male. Normally for the male, the testicular tissue structure, the epididymal sperm parameters, the serous sexual hormones, and the concentration of NPs in serum and testis need to be tested. For the female, sexual hormone levels in serum need to be measured.

16.2.4 IDENTIFYING THE RISK FACTORS

16.2.4.1 Difficulties in the Determination of the Mechanism of Nanotoxicity

Utmost NM-induced toxicity is over free-radical mechanisms, among which, group of oxidative stress is mediated by ROS formation. Fundamentally, free-radical mechanisms are challenging to expose at the molecular level as free activists are highly reactive and, in most cases, have an extremely short half-life. Most biologically significantly allowed activists are short-lived, making them tough to be straight noticed. ROS has a short half-life in the variety of about 10^{-9} s, and thus, without derivatization, are hard to be quarantined openly for structural identification. Thus, ESR spin trapping techniques to categorize the creation of ROS from chemical reactions are commonly used [51].

Biochemical and cell-based procedures are regularly used to reveal the acute and chronic toxicities produced by NMs. The profits of exhausting cultured cell lines are several, including reproducibility, the comfort of treatment with test material, and calm measurement of cytotoxicity and extra toxicological endpoints. It has been demonstrated that cultured cells have a significantly high sensitivity to the effects of NMs. However, associated with chemical reactions, it is more interesting to determine mechanisms of free radicals formed *in vivo* and the cells.

Otherwise, the association of outcomes from ESR studies with the cell-based results will deliver a summary for each NM that adds chemical and biochemical approaches. ESR spin trapping is the mere approach that offers through the

suggestion of the existence of free radicals and a means to recognize them. This makes it possible to study the energetic effects of free radicals in diverse classifications. ESR is extensively used in free radical research and is gradually used in mechanistic studies of free-radical generation in the existence of NMs. Mechanistic studies normally use ESR techniques for finding free-radical formation. For example, Yin et al. [52] determined the photo cytotoxicity of nano-TiO₂ in several different cell systems and found that the resulting toxicity was mediated by ROS. It is even more challenging to control the free-radical mechanism of NM-induced toxicity in a biological system, mostly *in vivo*.

16.3 RECOMMENDATIONS AND REGULATORY GUIDELINES FOR NANOPARTICLE TOXICOLOGY

Recommendations and decisions on feasibly toxic environmental materials should not be made by varied groups having a minor alternative of members with straight real experience of the difficulties of toxicity tests and their values. Common conclusions in such situations are invalid. For illustration, the advisory committee that mentioned the prohibition on DDT in the USA outvoted the dispute that the animal experiments performed to check for a carcinogenic effect were completely unreliable in both organization and evaluation [53]. Nowadays, everyone feels entitled to speak on toxic effects and the risk to health from environmental pollution; why cannot this task be left to the openly familiar experts functioning to loosen the difficulties of the field, as is the case in nuclear physics or oceanology.

Despite the wide range of feasible coverage situations and then a likely risk, the fixed guideline has been careful to emerge. One of the concerns here is that as of the depth of uses, from paints to cosmetics to medicines, many divergent monitoring outlines relate. The BRASS report has constantly informed that although the regulatory framework is capable of dealing with nano issues, the regulations are not well agreed to allocating with the facts of nano-sized materials. Presently, there is no ruling and guideline in NM toxicology at all regional and national levels. Conversely, it is a far from custom guidelines of productivity toxicological techniques that can powerfully identify factual toxicological factors for assessing NM toxicity since too many untouched factors. To take off, such systems or guidelines in NM toxicology are depended on the development.

16.4 SAFE BY DESIGN NANOMEDICINES

16.4.1 SAFETY OF NANOPARTICLES

NPs can be classified into different types according to the size, morphology, physical, and chemical properties. Some of them are carbon-based NPs, ceramic NPs, metal NPs, semiconductor NPs, polymeric NPs, and lipid-based NPs. NPs can have the same dimensions as biological molecules such as proteins. In living systems, they may instantly adsorb onto their surface some of the large molecules they encounter as they enter the tissues and fluids of the body.

The toxicity profiling of NPs is a highly demanding area of research world-wide in recent times. Natural NPs have been existing in the ecosystem for years, and they possess some mechanisms to cause less harmful effects among living organisms. NPs are tiny particles that can be inhaled or ingested and may pose a possible problem both medically and environmentally. The present research designates that exposure via inhalation and skin contact can result in NPs entering the body.

16.4.2 SAFETY OF NANOMEDICINES

The job of monitoring works needs to confirm that medicinal goods show a tolerable risk-benefit outline [54]. It is well known that “safe-by-design” may be an ultimate desire, while “safer-by-design” is a more faithful goal; varying materials to make them completely “safe” may not be likely and would not make intelligence if the necessary assets of the materials vanish in the process. To attain a harmless design of nanomedicines that requires an acceptance of material essential properties along with a thoughtful of the performance of these ingredients in living systems. According to Paracelsus, the dose creates the poison, but when distributing with NPs, one also wants to take into account, for instance, the size, shape, solubility, and crystallinity, and how these physicochemical properties add to the potential toxicity of the material in a request.

Modern investigation has proposed that the pretended corona of biomolecules adsorbed onto the surface of NMs may order the biological performance of these materials. The environment of the biocorona is possible to fluctuate depending on the gateway of entry. It has been advised that the biocorona could cover targeting ligands on NPs, thereby preventing specific interest of cells [55]. This surely takes suggestions for the scheme of NPs for the directed transfer of therapeutic or imaging agents. The rate value of fabricating multifunctional NPs for drug delivery with aiming and imaging moieties was just stressed. Thus, while the vision of nanorobots or nanomachines accomplished of theranostic in humans looked remote only a few years ago, this can become a truth earlier than estimated.

16.4.3 TESTING THE SAFETY OF NANOMEDICINES

Nanotechnology is measured as a technology of the prospect, with great potential in biomedical applications. It is becoming increasingly important in nano-diagnostics, drug delivery devices, and regenerative medicine. NPs have already been used for handling a variety of human diseases comprising infections, genetic disorders, and tumors. By some NP-based crops already used in nanomedicine and many more under development, it is unfavorably important that the potential risks are accurately assessed. Nanomedicine make humans continuously interact with NPs, and it is, therefore, crucial for public self-assurance and nanotechnology companies that suitable risk assessments are undertaken about health and safety. The European Commission has adopted the Action Plan “Nanosciences and nanotechnologies: An action plan for Europe 2005–2009”

[56] defining actions for the “immediate implementation of a safe, integrated and responsible strategy for nanosciences and nanotechnologies.” Besides, there are current discussions within European Commission committees [57], and within REACH (the Registration, Evaluation, Authorization and Restriction of Chemicals) competent authorities and the subclass on NMs as to hazard and risk assessment of NMs [58,59], originally, nanotechnologies and NMs were not involved in the scope of REACH.

However, the detailed structures and properties of NPs that may add to their potential toxicity are not fully addressed. Special consideration should be given to NPs’ physicochemical characteristics and the variations that may follow under local environmental conditions. Such things and their modifications include size, surface, chemical composition, bioavailability, solubility, agglomeration, dissociation, and adsorption of environmental substances, all of which may influence the ultimate toxicity of NPs. The main difficulty faced in testing NPs for human toxicity have been the shortage of appropriate standardized protocols. Conveying the NP dose as mass attention is probably not the best description for the dose-response connection. Regarding acquaintance to NPs, the strength and pertinence of prevailing technologies are not always perfect. The environmental things of NPs need to be estimated through the brilliant scenario formation, fabrication and use. Acquaintance and dose-effect representations may need to be adapted, taking into account their changing physicochemical properties over time and their relaxed humiliation. Improvements to the methodologies should take into account the following factors:

- a. Assessments for acquaintance should be considered that rapid dose in terms of number absorption and surface area, instead of mass concentration;
- b. NPs may agglomerate and disagglomerate in different environments, which could disturb their properties and these to be characterized;
- c. Impurities inside and adsorbed species on the surface of NPs may have important properties on risks, and these potentials should be taken into consideration;
- d. Biological procedures involving NPs, counting translocation, cellular uptake, and toxicological mechanisms, are quiet mostly unknown, and challenging methodologies have to address these potential ways of effect.

The FP7 project Nano TEST reports the requirements relative to the toxicological shape of NPs used in medical diagnostics. The complete aim of the project is to improve other testing strategies and high amount toxicity challenging protocols, *in vitro* and *in silico* methods are vital for the risk assessment of these NPs. To achieve this ambitious goal, the following steps should be carried out:

- a. To bring out a complete description of certain NPs, to explain their leading physicochemical properties.

- b. To study specific and nonspecific exchanges of NPs with molecules, cells, and organs and to develop *in vitro* methods that can detect the toxicological potential of NPs;
- c. To corroborate *in vitro* outcomes in short-term *in vivo* models, to train manifestation of particle effects in animals and humans, and to consider separable susceptibility in the reply to NPs.
- d. To achieve both structure-activity modeling and physiologically based pharmacokinetic modeling of NPs.

16.5 USAGE AND MANAGEMENT OF NANOPARTICLES

NPs now occur in nature, e.g., volcanic ash, ocean spray, and forest fire smoke, and they are extremely toxic. The present chemical NPs, e.g., carbon nanotubes, are also capable to create toxic effects. It is claimed that regular NPs are not extensive as a new occurrence since they come from day-to-day human activities like mining, cooking, and combustion of materials. NP readings have revealed that they have some distinct properties, i.e.

- a. Ultra-small-sized particles have exciting mobility and advanced transmission in living organisms if not measured,
- b. Produce more toxic effects per unit matched to superior particles of the same chemicals,
- c. Absorbed more quickly by cells, and
- d. Move quickly, polluting the environment through air, soil, and water, leading to damage of plants and animals [60].

Certain types of NPs are accountable for initiating incendiary reactions in the lungs, such as carbon black NPs, and cannot be measured as a uniform group of NPs as they relate and perform in dissimilar manners conditional upon numerous reasons [61]. The leading contests of NP procedure are the identification of toxicity-free NPs, authorized assessment of the ultimate health goods, and removal of elements from biological and environmental systems. Nanomedicine scraps are challengeable as they does not fall into the outdated classifications of drugs or medical devices. Still, the risk studies of NPs are complex because of the deficiency of data, techniques, and issues.

As for the environmental aspect, the excretory materials are primarily deferred in the air for long periods and affect respiratory disorders. The National Science Foundation and the Environmental Protection Agency are educating about the possible influence of NMs on the environment and their adverse effects. Public and moral issues are discussed about implantable nanodevices that strictly observer illness, privacy rights, and risk of cruelty. The values of nanodevices would be very high, which is an alternative social issue and one that disturbs the developing nations [62]. The proper concerns prove that NPs are non-toxic, eco-friendly, and easy to remove when toxic effects are produced in the body.

16.6 TOXICOLOGICAL PROBLEMS

16.6.1 TOXICOLOGICAL PROBLEMS RELATING TO CHANGES IN THE ENVIRONMENT

Giving to latest assessments, organics increase 250,000 different chemical compounds per annum to the two million now in survival. Approximately 300 of these innovative compounds are unrestricted every year into the environment, certainly controllable, several are not. Some of them are purely active, occasionally as the cautious and chosen effect of the chemical synthesis; for example, in the case of pesticides, disinfectants, food additives, and cosmetics and by no means least, drugs.

Several of these unwanted foods have features that are hypothetically injurious to man. Infection is common in some circumstances, even universal in others, and device processes are either useless or only moderately effective. Some toxicological assessment of environmental fluctuations must advance from the initial fact. Theoretically purely man-made, environmental toxins have lately been revealed to be regular elements of the environment, in precise of the atmosphere.

16.6.1.1 Long-Term Effects of Environmental Toxins

It is essential, and at the similar time extremely tougher, to assess the long-term effects of tiny doses of potentially toxic environmental materials on large occupied areas has become the major assignment of up-to-date toxicology. A certain list from a large number of synthetic environmental pollutants with essentially known toxic effects is known. In this situation, no description is full of lonely and restricted circumstances of harming due to the chance of an unclear environment. Even where the effects are convinced, they may show risky differences in magnitude, not only concerning the rate of occurrence but also their environment. For example, an assessable, but quantitatively immaterial creation of methemoglobin because of nitric oxides, or of carbon monoxide and hemoglobin due to carbon monoxide, denotes no real extreme risk under standard environmental situations, while the least rise in the rate of cancer is of the weightiest importance. Though, it will be seen with the one exception of asbestos, assumed to be carcinogenic.

There is no comprehensive technical origin for the announcement lately finished by a cancer researcher [63] and incidentally commonly overstated and misconstrued in the lay press to the effect that 90% of all diseases in man are attributable to biological hazards and 50% to conservational causes. They simply assist to feature the element that at the existent period disbelieved noxious things hugely outnumber those complete. On the other hand, it is of the way relatively terrible to rule out the chance of toxic risk in the case of the doubted effects. Besides, even where grievance to fitness has been confirmed with confidence, it has been tough to measure its range acceptably. Such worries illustrate not only environmental toxicology but also many other expenses of environmental

research. It is expected that the subsequent challenge at an enquiry of the origins will simplify a thoughtful of the location, as well as provide a base for the progress of operative methods to develop it.

16.6.1.2 Effect of Heavy Metal Contamination

Heavy metals are measured unique of the foremost sources of soil pollution. Heavy metal pollution of the soil is triggered by different metals, especially Cu, Ni, Cd, Zn, Cr, and Pb [64]. Certain heavy metals are of bio-importance to man and their day-to-day medicinal and nutritive grants had been increasingly suggested. Upraised Pb in soils may drop soil efficiency, and a very low Pb concentration may prevent vigorous plant procedures, i.e. photosynthesis, mitosis, and water absorption with poisonous signs of dark green leaves, lifeless of grown-up leaves, small shrubbery and brown tiny leaves, undersized greenery, and brown short roots. When heavy metals comprising agricultural surplus arrive in the marine atmosphere, it may become toxic to sea plants and animals. If compostable waste such as sewage sludge, municipal solid waste, and pig manure contain heavy metals, it may alter the composting practice by obstructing infectious growth. In the vermicomposting process, heavy metals disrupt soil larva life cycle.

- a. **On soil:** Soil impurity by heavy metals is of utmost essential anxiety all over the commercial biosphere. Heavy metal waste not only the outcome in hostile things on several factors but also source variations in the size, composition and activity of the microbial community. Metals that cause heavy metal contamination of the soil are mainly Cu, Ni, Cd, Zn, Cr, and Pb. The mud assets, sand guts, and pH have the most important impacts on the scope of the things of metals on biological and biochemical properties [65]. Chen et al. advised that heavy metals triggered a decline in bacterial species abundance and a virtual rise in soil actinomycetes or even drops in both the biomass and diversity of the bacterial communities in contaminated soils. Karaca et al. conveyed that the enzyme events are inclined in dissimilar ways by unlike metals due to the altered chemical attractions of the enzymes in the soil system.
- b. **On plants:** Some of these heavy metals, i.e., As, Cd, Hg, Pb, and Se, are not important for plants' growth, since they do not achieve any known physical utility in plants. On the other hand, other metals, i.e. Co, Cu, Fe, Mn, Mo, Ni, and Zn, are important elements essential for regular evolution and absorption of plants, but these elements can easily harm when their absorption is greater than ideal values. The convention of fertilizer to expand agricultural yield short of a kind with probable harmful things influence be difficult then the waste composts are most realistic to progress soils used to raise vegetables. The hazard of conversion of heavy metals from soil to humans must be trouble of distress [66]. Approval of

heavy metals by plants and consequent growth along the food chain is a possible threat to animal and human health. Plant roots are the main routes of the entrance of heavy metals in the food chain.

- c. **On the aquatic environment:** Heavy metals are extremely persistent, toxic even in trace amounts, and can bring severe oxidative stress in aquatic organisms. Thus, these are major pollutants in terms of ecotoxicology. Furthermore, metals are not subjected to bacterial degradation and hence persist always in the marine environment. Contamination of a river with heavy metals may lead to disturbing effects on the ecological stability of the aquatic environment, and the diversity of marine organisms may be limited with the extent of the contamination. Heavy metals discharged into aquatic systems are normally assumed to form particulate substances. External sediment is the essential reservoir or descend metals and add pollutants to the aquatic environments. A foremost segment of the design metals pronounced into the aquatic environment ultimately come to be linked with the environmental degradation by metals in areas where water quality processes are not overdone, however, bacteria in or near the deposits are adversely unnatural [67]. When heavy metals are gathered by an aquatic organism, they can be shifted to the upper classes of the food chain. Mercury is a significant impurity both because it influences marine bacteria and it is possibly harmful to humans.
- d. **On human health:** Consumption of food crops polluted with heavy metals is a chief food chain direction for human exposure. The food plants that are cultivated in extensive and nonstop farming have the ability to absorb elements from soil. The farming of such plants in polluted soil leads to a probable risk of the plant tissues carrying heavy metals. Heavy metals becomes toxic when they are not metabolized by the body and accumulate in the soft tissues.

Prolonged absorption of toxic metals has negative impacts on humans, and the related harmful effects start to progress only after some years of exposure. Cadmium (Cd) is a heavy metal toxicant with a specific gravity 8.65 times greater than water. The organs susceptible for Cd toxicity are the liver, placenta, kidneys, lungs, brain, and bones [68]. Depending on the severity of toxicity, the signs may vary from nausea, vomiting, abdominal cramps, dyspnea to muscular weakness.

16.7 METAL OXIDE NANOPARTICLES

During their production, use, and disposal, the engineered metal oxide nanoparticles (MONPs) are released into the environment and may pose a hazard to organisms in the environment. Figure 16.5 displays an overview of the possible pathways of release and fate of MONPs within the environment. The available mechanisms of MONPs release from products, articles, and technologies

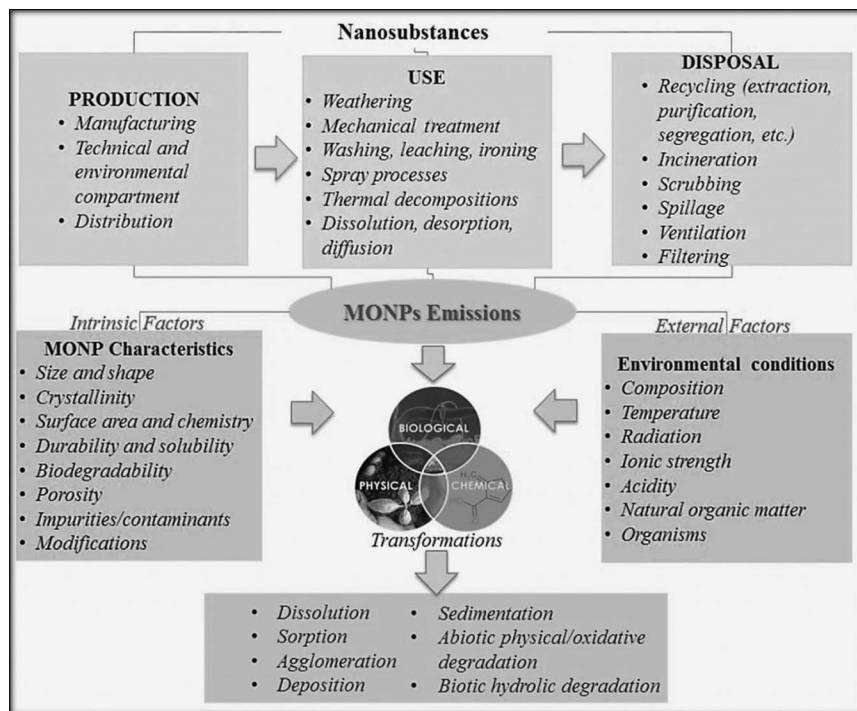


FIGURE 16.5 Possible pathways describing the release and fate of MONPs within the environment, while enlisting the potential sources of manufactured NPs emission; the key intrinsic and external factors together with the possible transformations are interactively related to the possible fate of the nano-substances in the environment.

can be segregated into passive diffusion, dissolution, and desorption into liquid media as well as photo-degradation, thermal-decomposition, mechanical treatment, or hydrolysis from matrix materials [69]. Although medical, cosmetics, and food-related products are excluded because of stringent safety regulations and dosing parameters, there are other nano-enabled products that release these particles such as thermosets, thermoplastics, coated surfaces, sprays, and textiles [70]. The removed fraction of MONPs may include different from the originally synthesized particulate matter (PM), dissolved fraction, vapors or transferred NPs to the solid compound. This fact suggests that the ecotoxicological investigations of NMs should be held with NPs released during their intended use. Some NPs such as CaO and MgO absorb CO₂ when exposed to air and becomes MgCO₃ and CaCO₃ that make the safety of these materials comparatively high [71]. In soil, the Brownian motion and gravitational force increased the chance for NP interactions with the surface of soil particles [72–73]. Other MONPs display adverse ecotoxicological effects which bring forward studies about the influence of the engineered materials on aquatic and terrestrial organisms.

The NP quantities in different environmental compartments differ depending on the NP type, production, release, life cycle, transformations, etc. For instance, the major sinks for ZnO NPs fell on freshwater sediments was predicted by environment concentration model to range between 30 and 4,800 $\mu\text{g/kg}$ for 14 years period (2000–2014) [74]. Other MONPs such as TiO_2 NPs (approximately 45% of the total NPs utilized), which are part of many commercial products and wastewater treatment plants, have influence mainly on aquatic ecosystems [75]. The predicted concentrations in sewage treatment plant effluent range from 5 to 15 $\mu\text{g/L}$ Ti, whereas in the sewage treatment plant, Ti content of the sludge varies between 1 and 6 g Ti/kg. Because of the wide use of CeO_2 as fuel material in agriculture and automobiles, the estimated concentration of CeO_2 NPs could vary depending on (i) soil depth and (ii) distance from the highway between 0.32 and 1.12 mg/kg [76]. With increase in the variety of nanotechnologies and engineered nano-products, the amount of emitted MONPs in the environment rises. Furthermore, the manufacturing or recycling processes could add different chemicals such as detergents or additives that could not be fully removed from the nano-substances, thus increasing their toxicity [77]. The external physical factors such as irradiation from sunlight may trigger photocatalytic activity which accelerates metal ion dissolution, phase transformation, or enhanced ROS formation. When entering the aquatic or soil ecosystem, chemical transformation processes such as degradation, dissolution, and alteration of the surface properties by precipitation or adsorption or desorption are expected to occur [78]. It is evident from the literature that oxidative stress and ion releasing were the drivers for many toxicological effects of MONPs. Not only the environmental (external) and MONP (intrinsic) characteristics but also the biology and physiology of the target organisms may be of substantial importance for risk assessment. For example, green algae *Raphidocelis subcapitata* appeared to be the most sensitive organisms toward ZnO, TiO_2 , and CeO_2 NPs as compared to *Daphnia magna* and fish embryo of *Danio rerio*, whereas the influence of NP zeta potential on the ecotoxicological test was comparably less [79].

The toxicological effects valued based on oxidative stress-related biomarkers of CuO and ZnO NPs on *D. magna* indicated changes induced by both metal ions released from the NPs and NPs themselves [80], while the influence of TiO_2 NPs on *D. magna* population in a multi-generation study revealed decreasing reproduction over generations resulting in population collapse after five generations [81]. Similar observations were presented by Muller et al. [82–86] who studied the mechanistic model of hatching of zebrafish eggs exposed to CuO NPs in a high-throughput screening system and described the toxic impact of dissolved copper on hatching. The *in vivo* effect of ZnO NPs at a concentration closer to the predicted environmental levels on *Ruditapes philippinarum* widespread in coastal habitats is related to significant toxicological effects (increased catalase and superoxide dismutase activity, DNA damage in hemocytes, etc.) because of ion release and changes triggered by ZnO NPs-specific features [87–89]. Table 16.2 summarizes some of the ecotoxicological effects of various MONPs on different ecosystems/organisms in different environmental compartments.

TABLE 16.2
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
Impact of Cu and CuO NPs on Plants					
CuO (~20)	200, 400, 600, 800, 1,000 mg/L	Growth medium with agar (treatment on germinated seeds)	Mung bean (<i>Phaseolus radiates</i>), and common wheat (<i>Triticum aestivum</i>)	• Decrease in seedling and shoot growth with an increase in NP concentration • In <i>P. radiates</i>, no adverse effect on shoot growth, whereas in <i>T. aestivum</i>, shoot growth was effected even at 200 mg/L concentration • Roots were more affected by NPs than the shoot.	[90]
CuO (30)	0.025, 0.25, 0.5, 1, 5 mg/L	Hydroponic (treatment on plants)	Waterweed (<i>Elodea densa</i>)	• Catalase and superoxide dismutase activities increases by 1.5–2 times • Stimulated photosynthesis up to 0.25 mg/L level, whereas suppressing it above 1 mg/L concentration	[91]
CuO (<50)	0, 5, 15, 30, 45, 60, 100, 200, 400, 600, 800, 1,000, 1,500, 2,000 mg/L	Petri plates (treatment on seeds)	Soybean (<i>Glycine max</i>) and chickpea (<i>Cicer arietinum</i>)	• A decline in root and shoot growth on above 100 mg/L concentration • A decline in root and shoot growth above 45 mg/L concentration	[92]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CuO (<100)	10, 100, 50, 1,000 mg/L	Petri plates (treatment on seeds)	Radish (<i>Raphanus sativus</i>), ryegrass (<i>Lolium perenne</i>), annual grass (<i>Lolium rigidum</i>)	• The DNA damaged was found to be increased (DNA lesions compound) with an increase in the concentration of NPs	[93]
CuO (30)	0.5, 1, 2, 5, 10, 20, 50, 100 mg/L	Growth media with agar (treatment on germinated seeds)	A flowering plant (<i>Arabidopsis thaliana</i>)	• A dose-dependent reduction in fresh weight, root length, and total chlorophyll • A dose-dependent increase in anthocyanin content, superoxide, and hydrogen peroxide • Loss of root gravitropism • Significant induction of genes related to oxidative stress responses, sulfur assimilation, glutathione, and proline biosynthesis	[94]
CuO (<50)	0.5, 1, 1.5 mM	Cotton pads shocked with growth media (treatment on seeds)	Barley (<i>Hordeum vulgare</i>)	• A dose-dependent reduction in shoot and root growth • A significant decrease in the GSH/GSSG ratio • Increase in hydrogen peroxide and lipid peroxidation with increased concentration of NPs	[95]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CuO (30–40)	680 ± 60,	Hydroponic (treatment on seeds)	Gibbous duckweed (<i>Lemna gibba</i>)	• Dose-dependent decrease in plant growth, and PS II activity	[96]
	1,004 ± 120,			• Inactivation of PS II reaction centers, a decrease in electron transport, and an increase in thermal energy dissipation	
	2,008 ± 340,			• Dose-dependent decrease in root length	
	4,051 ± 950 mg/L			• NPs were absorbed by roots and translocated to shoots	
CuO (43 ± 9)	100, 200, 500, 1,000 mg/L	Petri plates or hydroponic (treatment on seeds or germinated seeds)	<i>Elsholtzia splendens</i>	• A dose-dependent decrease in chlorophyll a, b, and total chlorophyll was observed	[97]
CuO (<50)	2.5, 10, 50, 100, 1,000 mg/L	Petri plate and hydroponic (treatment on seeds)	Rice (<i>Oryza sativa</i>)	• Accumulation of NPs in chloroplast	[98]
				• A dose-dependent decrease in thylakoid number per grana, photosynthetic rate, transpiration rate, stomatal conductance, the maximal quantum yield of PSII photochemistry, and photosynthetic pigment contents	
				• A dose-dependent increase in ascorbate peroxidase and superoxide dismutase	

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CuO (30–50)	10 mg/L	Hydroponic (treatment on the plant)	Waterweed (<i>Elodea nuttallii</i>)	• Ultraviolet (UV) radiation treatment increases the Cu concentration in shoots • UV radiation enhances the phytotoxic effect of NPs	[99]
CuO (40)	10, 50, 100, 150, 200 mg/L	Hydroponic (treatment on plants)	Common duckweed (<i>Lemna minor</i>)	• Increase in peroxidase, catalase, superoxide dismutase activity • Increase in lipid peroxidation • Inhibition of plant growth	[100]
CuO (<50)	3, 10, 30, 300 mg/kg	Pots with sand (treatment on seeds)	Common wheat (<i>Triticum aestivum</i>)	• Inhibition of root elongation by CuO NP (>10 mg/kg) • The exposure resulted in root hair proliferation and shortening of the zones of division and elongation	[101]
CuO (30 ± 10)	10, 200, 1,000 mg/L	Hydroponic (treatment on plants)	Transgenic cotton (<i>Gossypium</i> sp.) (Bt-29317), conventional cotton (Jihe321)	• Decrease in growth, development, nutrient content, indole-3-acetic acid (IAA), and abscisic acid (ABA) concentrations • Reduce the uptake of nutrients, such as B, Mo, Mn, Mg, Zn, and Fe, and inhibit the transport of Na and Mn in cotton plants • Enhance the expression of Bt-toxin protein in leaves and roots	[102]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CuO (20–40)	20, 50 mg/L	Hydroponic (treatment on seeds)	A flowering plant (<i>Arabidopsis thaliana</i>)	• Inhibit the seedling growth of different ecotypes (Col-0, Bay-0, and Ws-2) • Col-0 was most sensitive ecotype to NP among three	[103]
CuO (20–80)	20, 50 mg/L	Germination in culture dishes	A flowering plant (<i>Arabidopsis thaliana</i>)	• CuO NP was observed from root till seeds • CuO NPs (20 mg/L) were adsorbed and agglomerated on the root tips and meristematic zone • At concentration 50 mg/L, the root top was damaged • With the increasing of CuO NPs exposure concentration, the number of root tops decreased	[104]
Impact of TiO₂ NPs on Plants					
TiO ₂ (5)	300 mg/L	Pots (treatment on seeds and leaves)	Spinach (<i>Spinacia tolerances</i>)	• More than 60% increase in plant fresh and dry weight • The amount of Rubisco activase increased by 42%, whereas its activity increased 2.5 times compared to untreated samples	[105]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
TiO ₂ (25)	300 mg/L	Hydroponic (treatment on germinated seeds)	Corn (<i>Zea mays</i>)	• Leaf growth inhibition and transpiration via physical effects on root water transport	[106]
TiO ₂ (<100)	2,000, 10,000, 20,000, 40,000 mg/L	Petri plate (treatment on seeds)	Narbon vetch (<i>Vicia narbonensis</i>) and corn (<i>Zea mays</i>)	• Decrease in root elongation • The decrease in the mitotic index • Increase in aberration index	[107]
TiO ₂ (14, 25, 140)	100 mg/L	Hydroponic (treatment on the plant)	Rapeseed (<i>Brassica napus</i>) and common wheat (<i>Triticum aestivum</i>)	• Absorbed by plants, with Brassica having a higher capacity to the absorbed NPs (14 nm particle was absorbed more than 25 nm) • Moderate or no effect on plant growth • Accumulation TiO₂ NPs in roots with a primary diameter lower than 140 nm	[108]
n.a.	100, 200, 300 mg/L	Field (treatment on the plant)	Common wheat (<i>Triticum aestivum</i>)	• Titanium dioxide NPs at 0.02% increased different agronomic traits including gluten and starch content under water-deficit condition	[109]
TiO ₂ (15)	100 mg/L	Petri plates (treatment on seeds)	Flax (<i>Linum usitatissimum</i>)	• Reduction in root biomass and root length • Reduction in seed germination after 24 hours	[110]
TiO ₂ (21)	10, 100, 1,000 mg/L	Hydroponic (treatment on bulb with 2–3 cm roots)	Onion (<i>Allium cepa</i>)	• Concentration-dependent increase in genotoxicity	[111]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
TiO ₂ (90–98)	12.5, 25, 50, 100 mg/L	Hydroponic, (treatment on bulb with 2–3 cm roots)	Onion (<i>Allium cepa</i>)	• Concentration dependent increase in ROS • A concentration-dependent increase in genotoxicity	[112]
TiO ₂ (11.93–18.67)	0, 20, 40, 60, 80, 100 mg/kg	Pots with soil (treatment on seeds)	Common wheat (<i>Triticum aestivum</i>)	• Increase in root and shoot length with the treatment of 60 mg/kg or less • Decrease in root and shoot length above 60 mg/kg concentration	[113]
TiO ₂ (25 ± 0.64)	0, 100, 250, 500, 750, 1,000 mg/kg	Pots with soil (treatment on plant)	Tomato (<i>Solanum lycopersicum</i>)	• Up to a 250 mg/kg promoted the plant height, root length, and biomass • Lycopene content and fruit yield were maximum for 100 mg/kg • Chlorophyll concentration increases up to 750 mg/kg of the NP	[114]
TiO ₂ (<25)	0.01, 0.1, 1, 10 mg/L	Hydroponic (treatment on the plant)	Hydrilla (<i>Hydrilla verticillata</i>)	• Increase in catalase and glutathione reductase activity • 10 mg/L concentration has shown an increase in hydrogen peroxide level	[115]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
Impact of Other MONPs on Plants					
CeO ₂ (8)	500 mg/kg	Pots with soil (treatment on seeds)	Rice (<i>Oryza sativa</i>)	• Under NP influence, rice grain contains less Fe, S, prolamin, glutelin, lauric acid, valeric acid, and starch in comparison to control • NP could compromise the quality of rice grain • Reduction in Zn, Mg, Fe, and P levels in xylem sap	[116]
CeO ₂ (10 ± 3.2)	100, 500 mg/L	Hydroponic (treatment on germinated seeds)	Transgenic cotton (<i>Gossypium</i> sp.) (Bt-29317), conventional cotton (Jihe321)	• The decrease in indole-3-acetic acid and abscisic acid in the root of conventional cotton • Destruction of vascular bundles • Conventional cotton was more sustainable to CeO₂ NP stress in comparison to transgenic cotton	[117]
CeO ₂ (8)	0, 500, 1,000, 2,000, 4,000 mg/L	Soil contamination	Alfalfa (<i>Medicago sativa</i>), corn (<i>Zea mays</i>), cucumber (<i>Cucumis sativus</i>), tomato (<i>Lycopersicon esculentum</i>)	• In corn, tomato and cucumber seed germination was reduced at 2,000 mg/L • Promoted root elongation for corn and cucumber • Reduced root growth of alfalfa and tomato • Shoot elongation in all plant species	[118]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CeO ₂ (8 ± 1)	0–500 mg/kg	Soil contamination	Coriander (<i>Coriandrum sativum</i> L.)	• Increased shoot, root length, and biomass at 125 mg/kg • Higher Ce accumulation in tissue at 500 mg/kg • Increased ascorbate peroxidase activity in roots and catalase activity in shoots	[119]
CeO ₂ (<8 ± 1)	0, 62.50, 125, 250, 500 mg/L	Soil contamination	Rice (<i>Oryza sativa</i>)	• CeO₂ NPs' concentration in tissue increased with increased CeO₂ NPs but seedling showed no visible signs of toxicity • Reduced H₂O₂ generation in shoots and roots at 62.5 and 125 mg/L concentration • Augmented electrolyte leakage and lipid peroxidation were found in the seedling shoots grown at 500 mg/L • Changed ascorbate and free thiols levels and enhanced photosynthetic stress at 500 mg/L concentration	[120]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CeO ₂ (8 ± 1)	100, 200, 400, 800 mg/kg	Soil contamination	<i>Zea mays</i> plant	• The presence of organic matter in the soil increased the amount of Ce in the roots of the plant • The uptake of alginic acid-coated CeO₂ NPs was less than that of uncoated CeO₂ • Nanoceria in shoots was lower in organic matter enriched soil • Ceria was mainly present as CeO₂ inside the corn root tissue	[121]
CeO ₂ (8 ± 1)	0, 125, 250, 500 mg/kg	Soil contamination	Barley (<i>Hordeum vulgare</i> L.)	• With the increase of NPs concentration, the water and grain cerium content increased, whereas the number of spikes decreased • At 500 mg/kg CeO₂ NPs, the shoot biomass increased by 331 % • At 500 mg/kg CeO₂ NPs, the plant did not form grains • At 250 mg/kg concentration, Ce accumulated in grains and Ca, K, Zn, Mg, Cu, Al, Fe, P, and S in them increased	[122]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CeO ₂ (8)	0–500 mg/kg	Field (treatment on seeds) Pots with soil (treatment on seeds)	CeO ₂ (8) Common bean (<i>Phaseolus vulgaris</i>)	• 100, 400 mg/kg • Natural organic matter influences the behavior of NPs in the soils • Lower soil organic matter increased leaf cover area under NP influence • NP increased antioxidant enzyme activities in the aerial tissues	[123] [124]
CeO ₂ -citric acid coated (8+2) and CeO ₂ (8)	62.5, 125, 250, 500 mg/kg	Pots with soil (treatment on seeds)	Tomato (<i>Solanum lycopersicum</i>)	• Coated NP at 500 mg/kg increased CAT activity in leaves • At 250 mg/kg, coated NP increased total chlorophyll, chl-a, and chl-b • At 500 mg/kg, coated and bare NP increased stem length by 13% and 9%, respectively	[125]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
CeO ₂ (8 ± 1) and ZnO (10)	0, 400, 800 mg/kg	Soil contamination	Cucumber plants (<i>Cucumis sativus</i>)	• Both NPs had no impact on chlorophyll contents and gas exchange • CeO₂ NPs reduces the yield by 31.6% at 800 mg/kg concentration • At 800 mg/kg concentration, the bioaccumulation of Ce and Zn in the fruit was 1.27 and 110 mg/kg dry weight, respectively • Ce moved between tissues with water flow during transpiration	[126]
CeO ₂ (7) and ZnO (8)	0, 500, 1,000, 2,000, 4,000 mg/L	Petri plates (treatment on seeds)	Soybean (<i>Glycine max</i>)	• Genotoxicity recorded at 2,000 and 4,000 mg/L concentration • A new band in the roots' random amplified polymorphic DNA (RAPD) profile was observed, and therefore, both NPs affected the integrity of DNA but CeO₂ NPs caused the highest effect on genetic stability • The effect of CeO₂ NPs was attributed to the lack of Ce³⁺ ionic state to scavenge oxygen radicals leading to oxidative DNA damage	[127]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
ZnO (10)	750 mg/kg	Soil germination in a growth chamber	Symbiotic alfalfa (<i>Medicago sativa</i> L.) and <i>Sinorhizobium meliloti</i> (Gram-negative nitrogen-fixing bacteria)	• Most pronounced phytotoxic effects reducing the root and shoot biomass by 80%, and ionic Zn caused 25% reduction • ZnO NPs at 500 and 750 mg/kg reduce plant growth and dry biomass production • The aggregations of ZnO NPs in root nodules in long-term exposure dissolved Zn species that affected the nitrogen fixation, thus affecting soil fertility and plant growth • ZnO NPs' toxicity was less than ZnCl₂ and bulk ZnO	[128]
ZnO (<50)	500 mg/kg and 50 mg/kg	Soil contamination	Soybean (<i>Glycine max</i> L.)	• Reduced root and shoot lengths • The biomass generally decreased with the increase of ZnO NPs concentration • Inhibited volumetric stem growth • No seed formation at 500 mg/kg • Zn deposits were primarily found in vacuoles related to detoxification processes	[129]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
ZnO (90 ± 10)	0, 400, 800, 1,600, and 3,200 mg/kg dry soil	Soil contamination	Corn (<i>Zea mays</i>) and fungal inocula species (<i>Glomus versiforme</i> (Gv) and <i>Glomus calledonium</i> (Gc))	• A significant decrease in root colonization rate was observed at a concentration of 800 mg/kg and above ZnO NPs concentration • At concentrations above 400 mg/kg ZnO NPs, a significant decrease in shoot and root dry weights was observed • Gv and Gc increased the plant dry weight at all NPs doses • P, N, and K uptake in shoots and roots decreased at 1,600 and 3,200 mg/kg doses of ZnO NPs • Fungal inoculation decreased ZnO NPs bioavailability, Zn accumulation, and Zn partitioning in shoots, and increased ROS production and the content of mineral nutrients and antioxidant capacity	[130]
ZnO (20 ± 5)	10, 20, 50, 100, 200, 1,000 mg/L	Hydroponic (treatment on germinated seeds)	Ryegrass (<i>Lolium perenne</i>)	• Dose-dependent inhibition of root elongation • Above 20 mg/L concentration, a decrease in seedling biomass was observed	[131]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
ZnO (25)	400, 1,000, 2,000 mg/L	Pots or Petri plates (treatment on seeds and plant)	Peanut (<i>Arachis hypogaea</i>)	• Zn as a micronutrient can be delivered to plant through NP • Up to 1,000 mg/L, the NP promoted seed germination and growth vigor, whereas 2,000 mg/L was observed to be toxic for the plant	[132]
ZnO (~85)	200, 400, 800 mg/L	Hydroponic (treatment on plants)	Onion (<i>Allium cepa</i>)	• Showed an increase in cytotoxicity in root cells • An increase in DNA fragmentation reported • The observation indicated an increase in ROS and glutathione peroxidase production, whereas a decrease in catalase	[133]
ZnO (15.37)	100, 200 µM	Hydroponic (treatment on plants)	Common wheat (<i>Triticum aestivum</i>)	• Reduced photosynthetic efficiency • Increase in hydrogen peroxide and lipid peroxidation • Inhibition of antioxidant activity • Nitric oxide ameliorates the NP toxic effect	[134]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)			Concentration	Exposure Methodology	Investigated Plant	Impact	References
ZnO (20) and Al ₂ O ₃ (60)			20, 200, 2,000 mg/L	Petri plates (treatment on seeds)	Radish (<i>Raphanus sativus</i>), rape (<i>Brassica napus</i>), ryegrass (<i>Lolium perenne</i>), lettuce (<i>Lactuca sativa</i>), corn (<i>Zea mays</i>), cucumber (<i>Cucumis sativus</i>)	• Al₂O₃ NPs had no cytotoxicity except for corn whose root elongation was reduced by 35% • ZnO NPs inhibited root growth of corn and terminated root development of other 5 plant species • Supernatants of ZnO NPs enhanced the growth of rape and radish • Reduced seed germination and root growth by ZnO NPs in all plants • The inhibition occurred during the seed incubation process, rather than seed soaking stage • The phytotoxic effect was observed as 2,000 mg/L	[135]
Al ₂ O ₃ (n.a.)			100, 500, 1,000 mg/L	Petri plates (treatment on seeds)	Tobacco (<i>Nicotiana tabacum</i>)	• A dose-dependent decrease in the average root length, the average biomass, and the leaf count of the seedlings • Increase in expression of miR395, miR397, miR398, and miR399, with 1 % concentration of NP	[136]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
Al_2O_3 (13)	20, 200 $\mu\text{g/mL}$ and 2 mg/mL	Petri plates seedling	Corn (<i>Zea mays</i>), cucumber (<i>Cucumis sativus</i>), carrots (<i>Daucus carota</i>), cabbage (<i>Brassica oleracea</i>)	• The toxicity of particles reduced after being loaded with 10 and 100% monomolecular layer (MML) of Phen • While Al_2O_3 NPs inhibited root growth, 432.4% MML of Phen modified NPs were not toxic to the root growth • The surface characteristics of Al_2O_3 NPs were important for phytotoxicity of the particles • No toxic effect was detected • NPs were translocated throughout the plant tissues, detected in stem and leaves, accumulated on the surface of the root	[137]
Fe_3O_4 (20)	500 mg/L	Aqueous media growth	Pumpkin plant (<i>Cucurbita maxima</i>)		[138]
Fe_3O_4 (7)	62, 100, 116 $\mu\text{g/mL}$	Seed germination techniques	Cucumber (<i>Cucumis sativus</i>) and lettuce (<i>Lactuca sativa</i>)	• Low to zero toxicity on germination and root growth • Thicker roots were favored • In anaerobic bacterial consortium conditions, Fe_3O_3 NPs induced high biogas production	[139]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
Fe ₃ O ₄ (6)	0.064–0.64 mg/mL	Soil contamination	Lettuce (<i>Lactuca sativa</i>), radish (<i>Raphanus sativus</i>), cucumber (<i>Cucumis sativus</i>), spinach (<i>Spinacia oleracea</i>), tomato (<i>Solanum lycopersicum</i>), leek (<i>Allium porrum</i>), peppers (<i>Capsicum annuum</i>)	• Higher reduced germination at 0.64 mg/mL and lower at 0.64 mg/mL NPs • Some seeds (<i>Allium porrum</i> and <i>Capsicum annuum</i>) did not germinate under any conditions • The stabilizers (water and wastewater solvents) of NPs did not show any toxic effects	[140]
Fe ₃ O ₄ (25)	30, 100 and 500 mg/L	Soil contamination	Ryegrass (<i>Lolium perenne</i> L.) and pumpkin (<i>Cucurbita maxima</i>)	• Oxidative stress-induced increase in SOD and catalase enzyme activity in roots and shoots and block of aquaporins • Increased root elongation • No uptake and translocation in both plants • The content of Fe in shoots of both plants was not significantly increased	[141]
Fe ₃ O ₄ (10)	5, 10, 15, 20 mg/L	Petri plate and hydroponic (treatment on seeds)	Common wheat (<i>Triticum aestivum</i>)	• NP exposure did not alter germination, plant growth, and chlorophyll content • Plant exposed to NP showed a favorable response to prevent oxidative damage	[142]

(Continued)

TABLE 16.2 (Continued)
Effects of Various MONPs on Different Ecosystems/Organisms—Bacteria and Fungi; Algae, Crustacean, and Protozoan; Aquatic and Terrestrial Animals—Depending on the Nano-oxide Types and Doses

Nano Metal Oxide and Size (nm)	Concentration	Exposure Methodology	Investigated Plant	Impact	References
Fe ₃ O ₄ (17.7 ± 3.9)	20, 50, 100 mg/L	Hydroponic (treatment on seeds)	Corn (<i>Zea mays</i>)	• Germination index was observed to be higher with 20 and 50 mg/L NP treatment but decreases with 100 mg/L treatment	[143]
Fe ₃ O ₄ (6–7)	50, 500, and 200 mg/L	Petri plates (treatment on seeds)	Cucumber (<i>Cucumis sativus</i>)	• At high concentration (2,000 mg/mL), Fe₃O₄ NPs exhibit an increase in fresh biomass level and oxidative stress in the plant • In low concentration (50 mg/mL), the biomass level decreases	[144]
NiO (23.34)	25, 50, 100, 250, 500, 1,000, 2,000 mg/L	Petri plates (treatment on seeds)	Tomato (<i>Solanum lycopersicum</i>)	• NiO induces apoptosis in tomato root cells; increases in ROS, antioxidants, and mitochondrial membrane potential; triggers the release of caspase-3 proteases from mitochondria	[145]
NiO (<100)	87.8, 131.7, 197.5, 296.5, 444.4, 666.7, 1,000 mg/kg	Petri plates or pots with soil (treatment on seeds)	Barley (<i>Hordeum vulgare</i>)	• Increase in lipid peroxidation, superoxide anion radical, and cell deathThe decrease in leaf surface area, chlorophyll, and carotenoids	[146]
CdO (7–60)	2.03 ± 0.45 × 10 ⁵ particles/cm ³	Pots (treatment on the plant)	Barley (<i>Hordeum vulgare</i>)	• No change in total chlorophyll concentration, with a minor change in Fv/Fm with (3) treatment • Increase in total amino acids in all three cases with a maximum in (3) treatment	[147]

The high specific surface area resulted in strong adhesion of colloids and organic matter in water and soil leads to alteration of surface properties [121]. The current knowledge of the behavior of MONPs in different environmental compartments is presented in Figure 16.6. The figure also provides a glimpse into the possible fate of nano-substances when contacting some biotic and abiotic components. When released into the air, NPs precipitate with the rain, fall as water droplets, and deposit in the soil. Plants actively interact with soil, water, and air and are consumed by different animals and microorganisms. After successive interactions of MONPs with soil and water, the particles' composition changes, which may increase the toxicity of these substances. In an aqueous solution, MONPs form colloidal suspensions that can interact with aggregates and agglomerates [122]. This happens usually in marine ecosystems which are more alkaline, with high ionic strength, and have a wide variety of colloids and natural organic matters. Sinking in freshwater, NPs aggregates could accumulate in the sediment and influence benthic species (mainly biofilms).

Many properties of NPs like ROS generation, dissolution rate, etc. depend not only on the type of NPs but also on the surrounding environment [123] as well as the presence of organisms [124]. For instance, the complex organic agents produced by algae, bacteria, and plants may affect the rate of NP dissolution and biodegradability. MONPs may be a subject of trophic transfer and biomigration. For example, algae, daphnids, and zebrafish model the different

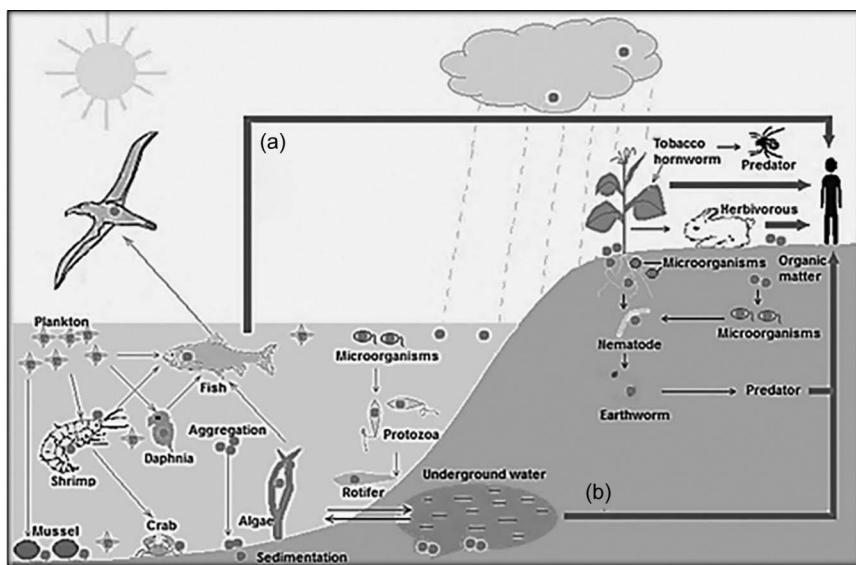


FIGURE 16.6 A schematic description of the possible pathways: (a) the fate of MONPs in the air, water, and soil and (b) potential harmful effects on humans; this interactive cycle includes the imaginable fate of nano-substances as part of the trophic transfer, bio-migration, or undergoing aggregation and migration.

trophic levels and feeding strategies in a water ecosystem. In a terrestrial environment, these are plants, their symbiotic interactions with microorganisms, nematodes, earthworms, and different predators. Thus, the impact of MONPs on communities of aquatic and terrestrial ecosystems and their horizontal and vertical trophic interactions have to be successively and simultaneously assessed. The risk assessment should include both exposure and bioaccumulation since bioaccumulation presents a straight way to process the impact of the bioavailability of NPs through possible exposure pathways [75]. Consequently, additional multi-generation and long-term examinations of diverse species in an ecosystem covering repeated assessment of the toxicological effects of soluble and non-soluble, bare, and surface-modified NPs are needed to be considered.

16.8 CONCLUSIONS

Nanotoxicology is presently the concrete way to advanced research methods in the edge between current technologies and safety goals of medical care. Development in nanotechnology will be unfavorably helpless on the capability of scientists and supervisors to successfully report suspicions and health concerns about the safety of NMs. Nanotoxicology investigation would similarly look forward to creating how protection can be evaluated for “next-generation” harvests and for distinct devices, such as microchip-based drug delivery systems, or active remote-actuated nanostructures that progress their assets or state during their operation. The succeeding era could be a turning fact in the industrial progress of a variation of new medical products found from nanotechnology.

Considering the advances of nanotechnology, several NMs (metallic, metal oxide-based, ceramic, carbon-based, polymeric, polymer-based, biomolecule-based, magnetic, and composites) are advanced along with several bulk varieties the matrix of NMs. Showing a huge number of NMs at *in vivo* level cannot offer the speed cost and faces virtuous anxiety. A huge amount of *in vitro* tests are accessible, and we need to conduct further experiment to be assured about the outcomes. In such condition, we need precise penetrating, quick, cost-operative, and easy-to-activate methodical tools in labs.

The future of transplant medicine is bright. Many of the strategies for encapsulating hydrophobic drugs that have shown promise in the field of oncology and cancer therapeutics apply to the transplant community. Toxicological profile concerns for commonly used immune suppressants can be addressed with proper encapsulation and NP stabilization strategies, allowing for increased therapeutic yields to reach its target. Surface modifications to creatively decorate the surfaces of NPs will enable increased delivery while avoiding nephrotoxicity and hepatotoxicity of common immune suppressants. Advancements in next-generation organ-on-a-chip, *in vitro* models, computational toxicology, and pharmacologic modeling can also be used to predict biocompatibility of newly designed small molecules and engineered NP formulations for transplant medicine applications.

AUTHOR CONTRIBUTIONS

GVSSS contributed to conceptualization, conception, and design of this study and writing the first draft of the manuscript. Authors GKT, MKE, and SRP helped us with the literature. Authors MPN and MC wrote major sections. Author MC did a formal analysis and formatting of the manuscript, including refining, reviewing, and editing. Rest of the authors read and equally contributed to this manuscript by supervision and validating.

DECLARATION OF COMPETING INTEREST

The authors declare that this is an independent research with no known commercial or competing financial interests or personal relationships that could have appeared to influence the work reported in this chapter. All authors have no potential conflict of interest.

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17 Current Guidelines and Regulatory Challenges, Insight into the Legal, Societal, and Ethical Issues of Nanomaterials

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17.1 INTRODUCTION

The term “nanomaterial” is generally used to refer a material that is smaller than 100 nm in at least one dimension (height, width, or length). Nanomaterials have unique properties, and hence, they find their usage in the areas of pharmaceuticals, drugs, electronics, etc. Various studies have revealed that these unique properties might be one of the main reasons for their potential health hazards. Such materials may require different testing methods for analyzing hazards, exposure, and risk assessment when compared to their bulk counterparts. The precise information about human exposure pathways for nanomaterials is limited. Data available on the studies of nanomaterials inhalation from *in vitro*, animals, and humans are insufficient. The major challenge that remains before the policymakers, at global level is to fix standard regulations which is possible only through research which is currently very limited and vast areas are still under investigations and not clearly defined. For a developing country like India, the limited investments in risk studies hinders the policy establishments to frame nanotechnology regulations (Sibal 2016). China is leading the nanotechnology-based research followed by the USA and then India (Web of Science (WoS) database published by the end of December 2016) (NBIC+ 2017). The position of India stands third among all other countries of the world, and this significant share of India in nanotechnology-based research is due to the active participation of India’s Department of Science and Technology (DST). DST under its Nano Mission funded nanotechnology-based research worth 1,000 crore INR over the years.

The risk assessment of nanotechnologies in India is not ranked high on the policy agenda as compared to the USA, China, or Europe. There has been numerous push of this issues by various individuals and organizations, but so far there has not been much significant policy action or research. Dhawan and Sharma have criticized the fact that under Indian Nano Mission Programme, more than 200 projects were funded in between 2001 and 2010, but very few articles and reports were found to have been published which is directly related to nanotoxicity studies (Dhawan and Sharma 2011). The International Conference on Nanomaterial Toxicology (ICONTOX), which is jointly organized by the Indian Institute of Toxicology Research and the Indian Nanoscience Society in 2008, was one among such rare exceptions (Fautz et al. 2015). The Science and Technology Ministry of India along with other funding agencies has also sponsored various research on nanotechnological environment, health, and safety (EHS) aspects. However, the initiatives did not lead to a significant research and policy output. Risk governance of nanomaterials is an important subject of research and negotiation in various international forums, which includes United Nations Educational, Scientific and Cultural Organization (UNESCO), Organization for Economic Co-operation Development (OECD), and other organizations. But so far there is no significant research or policy action has been taken up in India. The presence of nanomaterials is not a threat to society, but certain aspects of nanomaterials such as the mobility, increased reactivity, and certain properties nanoparticles are hazardous to the environment and living beings.

Realizing the importance of safe handling of nanomaterials, the Government of India's Nano Mission has constituted a nano-regulatory Task Force in 2016 under the dynamic chairmanship of the former director of DAE-IGCAR, Dr. Baldev Raj, for constituting a regulatory framework roadmap for nanoscience- and nanotechnology-based research in India. The Centre for Knowledge Management of Nanoscience and Technology (CKMNT) was formed based on the recommendation of the task force. CKMNT was jointly established by Nano Mission and the International Advanced Research Centre for Powder Metallurgy & New Materials (ARCI) under the guidance of Padma Shri Prof. G. Sundararajan. The team took up the initiative to prepare guidelines for workforces in R&D laboratories for the implementation of the best practices for safe handling of nanomaterials. The team consisted of eminent experts and published a comprehensive document on "Guidelines and Best Practices for Safe Handling of Nanomaterials in Research Laboratories and Industries" (Centre for Knowledge Management of Nanoscience & Technology 2016). Though much delayed, it is a very positive and welcoming step toward safer nanotechnology research in India. The document is based on comprehensive reports published by various regulatory bodies like Occupational Safety and Health Administration (OSHA), OECD, International Organization for Standardization (ISO), National Institute of Occupational Safety and Health (NIOSH), and other reports which were available in public domain (Centre for Knowledge Management of Nanoscience & Technology 2016). Recently, World Health Organization (WHO) has also developed certain guidelines with recommendations in terms of ethics and good laboratory practices on how to protect workers in a better way from the potential risks of harmful nanomaterials (Anon 2017). The recommendations given by such bodies help the researchers and professionals about occupational health and safety, especially those who are working in the field of nanotechnology. It helped them in making decisions about the best methods of protecting themselves against potential health risks, which may be caused due to improper handling of nanomaterials in their respective workplaces. It was extremely difficult for the international forums to publish the reports as no long-term adverse health hazards in human beings have been observed and reported so far. The publication of standard reports was delayed due to the fact that nanotechnology studies are relatively new and simultaneously are the ethical concerns too. Thus, it can be said that except for a few nanomaterials where human studies have already been conducted and is available, health recommendations should be mostly based on extrapolation of the *in vitro* evidences, animal or other studies taken from practical fields where nanomaterials exposure would have occurred. The challenge that remains in front of regulatory policymakers is that most of the research areas are vast in their own way and are still being investigated, which means results are still unknown. In the present scenario, stringent regulations may lead to an oppressive situation for further development, while under-regulation may lead to adverse health effects.

The current chapter deals with nanotechnology research, its outcomes in India, and its comparison with other countries of the world along with discussion on the societal impacts of nanotechnology, its potential risks and benefits for developing

countries, and its societal justice and civil liberties. This chapter also summarizes the ethics, guidelines, and good laboratory practices which have been framed by the Department of Science and Technology in India for the safety of its scientists and engineers.

17.2 STATUS OF NANOTECHNOLOGY IN INDIA

17.2.1 RESEARCH INITIATIVES

To promote nanotechnology in India, recently the Indian Government has funded more than 500 research projects throughout the country through various schemes. The data can be represented in the form of a graphical chart (Figure 17.1), which shows the distribution of projects over regions. From the figure, it can be seen that 42% of the funded projects have been executed by the southern states of India followed by the northern states and so on. Overall, the country's participation in nanoscience- and nanotech-based R&D is really commendable. The Indian states like Tamil Nadu, Karnataka, Maharashtra, West Bengal, Delhi, and Uttar Pradesh are involved in a comparatively good number of research projects mostly in the areas of nanoscience and nanotechnology (Samal and Manohara 2019; Anon 2009; Gupta 2008).

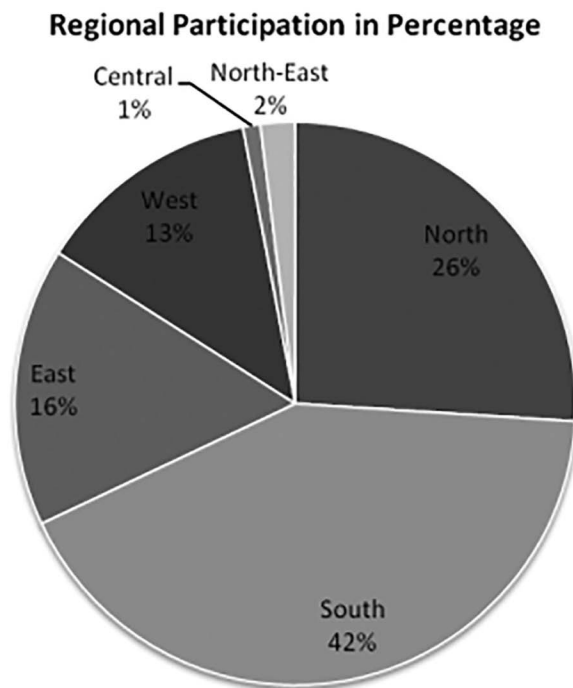


FIGURE 17.1 Participation of states in nanoscience and nanotechnology research in India.

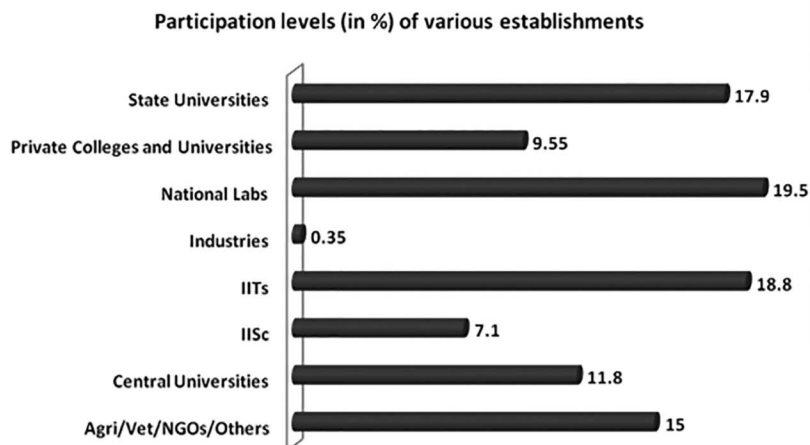


FIGURE 17.2 Participation of various establishments in the field of nanoscience and nanotechnology research in India.

The participation of Indian institutes in the field of nanotechnology and nanotechnology research is shown in Figure 17.2. From this figure, it is evident that (i) 19.5% of the total projects have been implemented by research organizations like DRDO, DST, DBT, CSIR, ICAR, ICMR, and MeitY; (ii) 18.8% of the overall projects have been executed by various Indian Institute of Technology; and (iii) 17.9%, 15%, 11.8%, 9.55%, 7.1%, and 0.35% of the projects have been carried out by state universities, agriculture-veterinary-NGOs, central universities, private colleges and universities, IISc, and industries, respectively.

17.2.2 EDUCATIONAL INITIATIVES

Universities, colleges, and institutions have started various undergraduate and postgraduate courses to strengthen the base of nanotechnology in India. The courses have been funded by the government under various schemes. More than 50 postgraduate and 10 undergraduate courses have been started by universities and institutions across India. Out of 60+ degree courses, 17 courses have been supported by the government of India. More than 50% of the degree courses have been initiated in the southern part (Andhra Pradesh, Telangana, Tamil Nadu, Karnataka, and Kerala) of India. Apart from that, many leading universities are offering PhD courses and electives in the domains of nanotechnology and allied disciplines (Samal and Manohara 2019; Anon 2009; Gupta 2008).

17.3 OUTCOMES OF NANOTECHNOLOGY RESEARCH

A number of key technologies and proof of concepts have been developed by the research groups due to a good amount of funding to the nanotechnology community of India from the government, especially under the Nano Mission of DST.

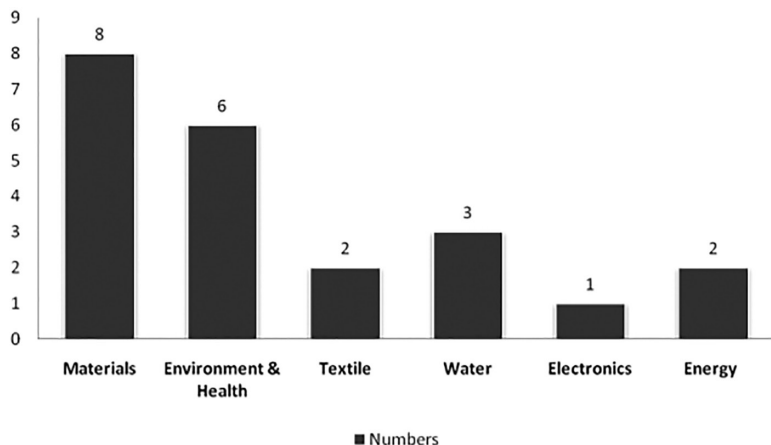


FIGURE 17.3 Outcomes of nanotechnology research. (Annual Reports of DST, 2009–2017, New Delhi, India.)

Some of the key areas are material synthesis, water, health, environment, electronics, and textile (Figure 17.3). To strengthen the Indian nanotechnology sector, the agencies have funded multidisciplinary groups for the development starting from low-cost nano-enabled technologies in materials to various other domains such as electronics and interactive nanotechnology education to name a few. These details are presented in Figure 17.4 (Samal and Manohara 2019; Kumar 2014; Anon 2019). Various government initiatives actually dominate the nanotechnology landscape in India, and the subsequent investments lead to a steady rise in global publication rankings. This also leads to increase in the number of scientific collaborations and the number of institutions involved. It was found that this growth is mainly rooted in fundamental research and public research institutes (Beumer and Bhattacharya 2013).

17.4 SOCIETAL IMPACTS OF NANOTECHNOLOGY

The societal impacts of nanotechnology may refer to the potential benefits and challenges for the introduction of nanomaterials, devices, and processes on society and human interaction (Babatunde et al. 2020). This can be termed as nanotechnology's health, social, and political impact. Most of the applications of nanomaterials are still speculative and are rather a matter of debate on the positive and negative effects of them. Apart from the risks associated with toxicity to human health and the environment due to first-generation nanomaterials, the domains of nanotechnology have broader societal implications and social challenges. Social scientists have suggested that the social issues related to nanotechnologies should be understood and assessed not only as “downstream” risks or impacts but also the challenges should be factored into “upstream” research and decision making to ensure societal technology development. Many social scientists and organizations in civil society suggest for public participation in technology assessment

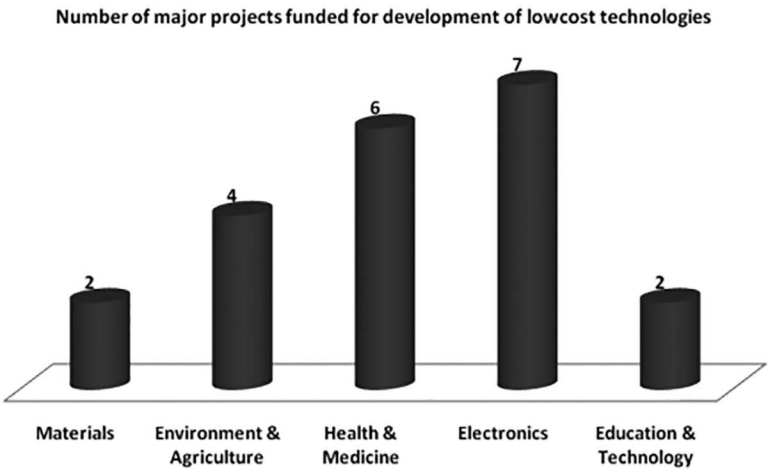


FIGURE 17.4 Projects funded for the development of nanotechnology.

and governance for discussing the social impacts. Eminent experts suggest that nanotechnology will build incrementally and will gear up to a pace to drive a nanotechnology revolution which will radically reshape our economies, our international trade, labor markets, international relations, civil liberties, social structures, and broad relationship with the natural world. The people who are concerned with the adverse effects of nanotechnology suggest that it will simply aggravate issues obstructing from existing socio-economic inequity and asymmetrical distributions of power, creating greater inequities between rich and poor through an unavoidable nano-divide. Experts suggest that nanotechnology has the potential to destabilize international relations through a nano arms race and the increased potential for biological weapons which will provide the mechanism for surveillance with significant implications for civil liberties. Critics believe that the barriers may break between living and non-living beings through a high-powered nano-biotechnology revolution.

17.5 POTENTIAL BENEFITS AND ASSOCIATED RISKS

Nanotechnologies may provide innovative solutions for the millions of people in developing countries in the area of health, hygiene, energy, space, defense, infrastructure, health, education, and climate change (Das et al. 2019; Samal and Bharati 2019; Uv 2018; Carmalin and Harjeet 2016). Many developing countries, for example Bangladesh, Chile, Costa Rica, Malaysia, and Thailand, are investing an appreciable amount of resources in the research and development of nanotechnologies. Emerging economies such as South Africa, India, China, and Brazil are spending huge resources annually on R&D, and are radically increasing their scientific output as demonstrated by their increasing numbers of publications in peer-reviewed international journals (Porcari et al. 2019). It is evident from Figure 17.5

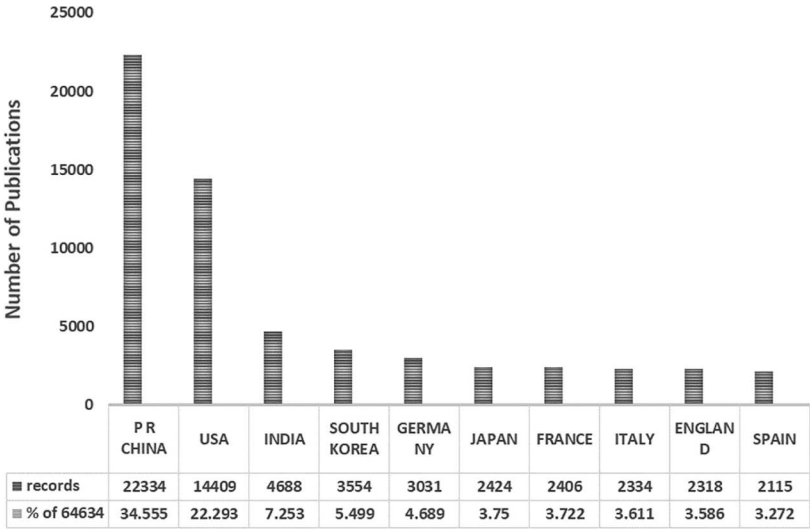


FIGURE 17.5 Recent data on top ten global publications on nanomaterials. (Source: Result analysis feature of Web of Science.)

that China is the leader in publications related to nanotechnology and is followed by the USA and India on the second and third spots, respectively. Standard nanoscience and nanotechnology regulations can minimize some of the risks specifically in the developing countries like India, and it is essentially important because of various social factors. Though all nanoparticles are not toxic, it is important that risk assessment, risk communication, and risk management are thoroughly laid down at industrial level as an essential standard for compliance. The risk assessment can be done through hazard identification, hazard characterization, hazard exposure, and hazard management (Mishra et al. 2019).

Potential opportunities of nanotechnologies can address global challenges like energy efficiency, security, defense, water purification, medicine and pharmaceuticals, agricultural and food production, nutrition, and information and communications technologies. Nanotechnologies are already incorporated in products which are in the market like water filter, cosmetics, light-weight and high-strength composites, and communication devices. Introduction of nanotechnology-based products in developing countries poses questions about the environmental, health, and societal risks which have major linkages between nanotechnology and product development. Protection of workers' health and the environment, and their safety in developing countries often suffers from a series of factors that include robust environmental conditions, human health, and poor safety regulation and enforcements. Physical infrastructure and trained human resources in the domain of nanotechnology are missing to control the impacts on EHS aspects. Greater uncertainties over the impacts of nanotechnology are the major challenge for social institutions, governments, civil societies, companies, and the common people in developing countries as compared to developed countries to make decisions about the governance of nanotechnology.

Nanoscale materials can enhance the strength and durability of products. Path-breaking nanotechnology applications may result in a huge demand for certain products. International organizations have initiated global dialogue on mechanisms that will allow developing countries to participate and respond to such changes.

17.6 CIVIL LIBERTIES AND SOCIAL JUSTICE

Most of the nanotechnology-related patents are concentrated among few leading multinational corporations. This has led to an apprehension that developing countries will have limited access to the infrastructure, funding, and human resources required to support nanotechnology research and development which has created a feeling of insecurity and inequalities (Karim and Munir 2014). The agriculture and food industries demonstrate the highest number of nanotechnology-related patents. Patents on robust seeds, plant material, life science, and other agri-food techniques are already concentrated among a few leading corporations. This is anticipated to increase the cost of farming, by increasing farmers' input dependence. This may not be helpful for farmers living in developing countries. Producers of agricultural and food products in developing countries may be affected adversely by the replacement of natural products (including rubber, cotton, coffee, and tea) by nano-modified products. These natural products are important export products for developing countries as the farmers' livelihoods depend on them. It has been studied that the substitution with industrial nano-products could negatively impact the economies of developing countries which were traditionally relied on the export of crops.

17.7 ETHICS

Nanotechnology is still considered as one of the new emerging technologies, and it has got a great potential to have impact on day-to-day life and is widely used nowadays. As nanotechnology is a new technology, proper controlling and regulating measures are needed. Though there are various concerns on the efficacy, effectiveness, cost, and safety of nanomaterials, there is limited consideration on misconduct and hence nanotechnology may be misused too. In preventing the misuse of nanotechnology, the ethical issue plays an important role. It is known that every technology also has two sides which need to be understood and managed well to extract the maximum output. The possible adversities and consequences of any new technology can be dangerous if it is not used legally or ethically without any control. So, without ethical control, various forms of unwanted problems may emerge from the uncontrolled use of it. There must be a proper education, guideline, and code of conduct for dealing with it. International collaboration for establishing ethics in nanotechnology research is of outmost importance. Both scientific and humanistic regulations are required in order to achieve a sustainable management for the implementation of new technologies such as nanotechnology (Toth-Fejel 2004). As the study of nanotechnology is a newer area, its ethical problem is also a new thing and may get forgotten and misused, but it is an important issue in the present era. Essentially both ethical and legal controls do have a very important role to pay

in order to harmonize the use of new emerging nanotechnology-based fields such as nanomedicine, nano-electronics, and nano-insecticide. Various publications on nanotechnology repeatedly sensitize us that ethics is very important and it must be a very important part of it. On analysing articles from the fields of natural sciences, humanities, and social sciences, it can be concluded that “ethics” means different things to different people (Toumey 2019) and there are two main reasons for this disunity. The first one is that professional ethicists generally speak a specialized language. Such people can tell us that Aristotle, Bentham, and Kant created three distinct frameworks for ethics, and what it means when a person chooses one over the other two. But at the same time, there have been multiple venues in which anyone can express his or her own understanding of nanotechnology ethics. Scientists, engineers, historians, policymakers, etc. should speak and offer their views in a similar and easy language which is more clear and effective, and the views of these populations tend not to be grounded in the language of the professional ethicists.

17.8 GUIDELINES

The health impact of nanomaterials depends on their effective use. A debate that always exists in nanotechnology community is that whether it poses health benefits and hazards to mankind (Méndez-Rojas et al. 2014; Karim 2012; Linkov 2009). Nanotechnological health impact can be classified into two aspects, namely, the potential for nanomaterial innovations for medical applications to identify and address diseases and also the health hazards posed due to exposure to nanomaterials. CKMNT in India has provided clear guidelines for handling nanomaterials and have divided it into three sections, viz. identifying hazards, pathways and common tasks that can result in exposure, and exposure control strategies (Centre for Knowledge Management of Nanoscience & Technology 2016).

17.8.1 HAZARD IDENTIFICATION

It is the very first step that must be followed in determining the risk and exposure, and it basically deals with identifying nanomaterials and their associated processes which may lead to toxic, physical (e.g. strong electromagnetic flux), and physicochemical hazards. Special care should be taken while assessing the risks associated with nanomaterials in identifying the effect of size, shape, morphology, and surface chemistry on the toxic effects caused to the various organs of our body. The primary hazard categories are presented in a simple form in Table 17.1, and it may be considered when assessing risks associated with nanomaterials.

17.8.2 EXPOSURE PATHWAYS AND COMMON TASKS

Humans get exposed to nanoparticles through various modes such as inhalation, ingestion, and dermal absorption. The state of nanomaterials also decides their exposure potential. For example, nanoparticles in suspension pose a comparatively less inhalation hazard potential than in its powdered form. Some of the exposure pathways and suggested prevention methods are summarized in Table 17.2.

TABLE 17.1**The Factors and Details Associated with Various Hazard Threats Posed by Nanomaterials**

Factors	Details	References
Surface charge	Cationic nanoparticles are more toxic followed by anionic and then neutral nanoparticles.	Villanueva et al. (2009)
Surface chemistry	It may sometime play a role in the generation of free radicals on nanoparticles, and this may result in the influence of toxicity and surface reactivity of the injected particles.	Sharifi et al. (2012)
Particle shape	Various studies have revealed that exposure to fibrous particles like asbestos and tubular structures such as carbon nanotubes (CNTs) may lead to fibrosis, cancer, inflammation, and lesions in the lungs.	Oberdörster et al. (2015); Acheson et al. (1985)
Particle size	Nanoparticles have a fair chance of accumulating in the lungs and can cause damages by the process of translocation to other organs through the blood. Nanoparticles have also been found to penetrate the membrane barriers like the human skin for serious damages.	Lu et al. (2014)
Solubility	Generally, poorly soluble inhaled nanoparticles have been reported in various studies to have caused oxidative stresses. This may lead to inflammation, fibrosis, and cancer.	Khanna et al. (2015)
Toxic hazards	The guidelines on exposure of nanomaterials like the threshold limit values (TLV) or permissible exposure limits (PEL) help in determining safety precautions. E.g. for materials with TLV or PEL less than 50 ppm, utmost care must be taken while handling and preferably under a proper fume hood. The Material Safety Data Sheet (MSDS) or container label is helpful in identifying toxic substances present inside.	Kuempel et al. (2012); Marsh
Fire hazard	Flash point of a chemical/nanoparticle is a measure of its flammability, and any chemical with a flash point below 93°C should be considered as a fire hazard.	Government (2008)
Explosion hazard	Factors such as the minimum ignition energy (MIE) and the minimum exposable dust concentration (MEC), which is also known as lower explosion limit (LEL), can give an idea about the flammability and explosive nature of nanomaterials. Because of their large surface to volume ratio, nanopowders get easily charged electro statically which increases the risk of ignition.	Heitbrink, Lo, and Dunn (2015); Yuan et al. (2014); Wu, Chang, and Hsiao (2009)

17.8.3 EXPOSURE CONTROL STRATEGIES

Exposure control strategies also are a very important part of the guidelines. Some of the strategies that can be followed for controlling exposure to nanomaterials are outlined in Table 17.3.

TABLE 17.2**Exposure Pathways and Safety Measures (Centre for Knowledge Management of Nanoscience & Technology 2016)**

Type of Exposure	Pathway	Safety Measures
Dermal	Nanoparticles has the capacity to migrate through the skin and circulate in the body.	Wearing of gloves and lab coat while handling the nanoparticles.
Ingestion	If good hygiene practices are not followed while having food stuffs, nanoparticles might be absorbed and transported within the body by the circulatory system.	Prohibition of eating and drinking inside laboratories and at the same time the spills of nanoparticles should be quickly and properly cleaned up.
Inhalation	Nanoparticles may enter inside the trachea, bronchioles, or alveoli of the lungs by respiratory absorption through the mucosal linings.	Nanoparticles are to be handled in the form of solution or on a substrate, i.e. not in a form which is easily airborne, e.g. powdery. Use of air filters N100 or N95 is highly recommended.
Injection	Exposure due to accidental injection, e.g. skin puncture, when working.	Wearing of gloves and lab coats is essential. Application of standard practices for working with sharp objects.
Ocular	The exposure to airborne nanoparticles placed near the eyes or accidentally splashed onto the eyes or transferred from the hands during rubbing of the eyes.	Wearing of safety glasses, goggles, and full-face piece respirator. Avoid wearing contact lenses at workplace.

17.9 GOOD LABORATORY PRACTICES

Ensuring good laboratory practices is outmost essential in handling nanomaterials properly, thereby safeguarding the researchers' life. CKMNT in India has also proposed few good lab practice steps which if followed can be of great help in handling nanomaterials in laboratories and industries (Centre for Knowledge Management of Nanoscience & Technology 2016). A combination of various aspects of maintaining good laboratory practices are summarized in Table 17.4.

17.10 FUTURE SCOPE

For nanotechnology-based research domains, the vision involves understanding and manipulating the matter at atomic scale. The nanotechnology domains are quite promising and risky too because nanotechnology-based research is leading to unprecedented understanding and control over the fundamental building blocks of all physical things. Such studies and experiments may lead to path-breaking changes in various areas, starting from vaccines to computers,

TABLE 17.3**Exposure Controlling Strategies (Centre for Knowledge Management of Nanoscience & Technology 2016)**

Controls	Safety Measures
Engineering controls	Nanoparticle exposure can be controlled by application of the following protection measures: • Ensuring proper enclosure of the source. • Usage of high-efficiency particulate air (HEPA) filters for capturing airborne nanomaterials and usage of proper exhaust ventilation system in the workplace. • The ventilation systems must be monitored routinely, and proper records of them must be maintained for their effective usage. • Proper usage of glove boxes, glove bags, fume hoods, etc. when there is a chance for aerosolization. • Usage of fume hoods or other local exhaust devices for exhausting tube furnaces and/or chemical reaction vessels. • In order to collect nanomaterials out from a reactor, airtight and sealed containers should always be used. • While evaporating a solvent from a colloidal dispersion using a distillation system, the setup should be enclosed within an explosion-proof enclosure. • If possible, remote control-based equipment setup can be used for nanomaterial production.
Administrative (procedural) controls	It usually comprises a supplementary approach when other control methods failed to achieve the desired control levels. Some control strategy includes: • Educating researchers by giving proper training and implementation of instructions for handling nanomaterials safely and following standard operating procedures (SOP), personal protective equipment (PPE), hazards and toxicity, waste handling, emergency procedures, engineering controls and equipment maintenance, exposure monitoring, applicable regulation, environmental release/shipping/customer protection, and proper labeling cum handling of nanomaterials waste. • Following good housekeeping protocols and practices in nanomaterial handling laboratories can essentially minimize the risk of exposure. Various strategies such as cleaning of all working surfaces potentially contaminated with nanoparticles at the end of each day by using industrial vacuum cleaner equipped with HEPA filters and properly labeled as “For use with nanoparticles only.” Such devices must be used exclusively only for this purpose only. Dry sweeping or compressed air usage must be prohibited. The disposal of used cleaning materials should be done as per the hazardous-waste procedures. • Good work practices such as proper handling of nanomaterials in solutions or attached to substrates must be followed in order to minimize the chances of airborne release of nanomaterials. The material safety data sheet (MSDS), if available, or other appropriate references must be consulted. • Ensuring proper medical surveillance by the effective usage of health surveillance program which might indicate whether exposure is occurring or not. Regular health monitoring program is highly recommended along with periodical medical surveillance of pulmonary, renal, liver, and hematopoietic functions.

(Continued)

TABLE 17.3 (Continued)
Exposure Controlling Strategies (Centre for Knowledge Management of Nanoscience & Technology 2016)

Controls	Safety Measures
Administrative (procedural) controls	• Effective record-keeping practices must be followed to maintain safe and healthy workplaces. Records of the areas such as induction and training programs, servicing and testing of equipment, workplace monitoring, risk assessments, health surveillance, work place engineering control maintenance, work-related injuries and illnesses, daily checks and examinations, and having disposal records need to be properly maintained. • Proper workplace monitoring can help identifying nanomaterial risk. The monitoring methods may include scanning mobility particle sizers or other means of particle collection systems as well as monitoring the particle concentrations at the workspaces using different types of particle counters. • Ensuring proper nanomaterial storage as different nanoparticles may require different types of storage containers in order to ensure workplace health and safety. Such containers must be selected depending on the different granulometric characteristics and reactivity of particles. E.g. certain nanomaterials such as metallic nanoparticles may get easily oxidized and may get exploded on exposure to air. Such nanomaterials must then be stored under inert gas/liquid bath.
Personal protective equipment (PPE)	Wearing of imperfect PPE kits may lead to nanoparticle exposure. A good PPE kit must include closed-toed shoes made of a low-permeability material, long-sleeved shirt, nitrile gloves with extended sleeves, long pants without cuffs, full-face shields, respiratory air filters (N100 recommended or N95), chemical splash goggles, and safety glasses.
Waste disposal	It is one of the key steps in controlling the exposure of nanomaterials. Nanomaterial residues of milligram range can be easily poured in sealed containers. It then needs to be labeled properly as per guidelines and disposed of following standard protocols. Every organization or industry dealing with nanomaterial must have a predefined hazard area, and only in such places, the nanomaterial marked for disposal should be stored. For powdery and colloidal nanomaterials of higher amount, it should be treated as hazardous chemical waste. For particles that are more water soluble, the rules according to the toxicity class of the macroscopic material must be followed. It is generally not advisable to put or dump engineered nanomaterial waste in a regular dustbin or drain. The collected nanomaterial wastes should be enclosed in hazardous waste containers with secured caps or covers, labeled properly by mentioning “Contains Nanomaterials.”

TABLE 17.4**Good Lab Practice Protocols**

Categories	Good Practice to be Followed
Locating the emergency equipment	Everybody working in a lab or at workplace need to know the exact location of emergency equipment like fire extinguishers, safety showers fire alarms, etc. They should also know how to use it judiciously and in a proper way.
Hygiene	There are various aspects in managing a good hygiene, and some of them can be summarized as below: • Several studies have been reported where it was found that humans may get exposed to nanomaterials through ingestion process while consuming food stuffs and beverages or even applying cosmetics and as such all these activities must be prohibited. To suck liquids, direct mouth suction for pipetting or siphoning should also be avoided. • The hands play a negative role in nanoparticle exposure through ingestion and dermal contact. So, to minimize this, the hands must be frequently washed. Wearing of appropriate gloves is hence preferred. • Leaving the laboratory or workplace with used gloves may unwantedly contaminate day-to-day usage things like doorknobs, mobile phones, computers, etc., and it must be avoided.
Labeling and signage	Labeling of nanomaterials plays a great role in their proper handling, thereby ensuring human safety. Nanoparticles must be stored in a well-sealed container which can be opened easily and with minimal agitation of the contents and labeled properly by following the below procedures correctly. • The well-sealed containers containing nanomaterials must be labeled in such a way that identifying the nanomaterials is easy. For doing so, the containers must be labeled by avoiding abbreviations/acronyms and including the term “nano” in descriptor (e.g., “nano-zinc oxide nanoparticles” rather than just “zinc oxide”). If possible, the hazard warning symbol and the chemical concentration level should also be included in the label, if it is known. • While leaving some operations unattended (if so required), it is always advisable to post signs on the work table or work place which can communicate appropriately about the precautions and warnings which may lead to the release of hazardous chemicals/nanoparticles and may also result in facility failures. If possible, details must also be provided to tackle it in case of accidental release of hazardous chemicals.
Cleaning procedures and spills	It is another vital aspect in ensuring a good laboratory practice. The following steps must be followed: • During cleaning operations, precautions must be taken in case of any nanomaterial exposure, and one must always be vigilant while cleaning process is on. • Wearing of appropriate gloves is essential while working in a fume hood and handling materials. Once the job is over, the fume hood must be cleaned properly. • Monitoring the concentration of nanomaterials present in the lab air while cleaning up the facility. • In order to ensure a better overall protection, respirator masks must be used whether working outside a closed or an open fume hood.

(Continued)

TABLE 17.4 (Continued)
Good Lab Practice Protocols

Categories	Good Practice to be Followed
Cleaning procedures and spills	• Contaminated materials like used gloves, used wet wipes, and used nanomaterial containers must be disposed of as chemical waste following necessary protocols. • The exposed surfaces and the instruments/materials should be cleaned using wet wipes and HEPA filter–assisted vacuum cleaning systems. HEPA filters provide a better protection by not allowing the nanomaterials move out from the vacuum dust chamber. Vacuum cleaners with HEPA filters must be selected in such a way that the system inside never bypasses the HEPA chamber in case of choking. • Energetic cleaning methods such as using compressed air or dry cleaning should be avoided. • The spilled nanomaterials must be sealed in a tightly closed container after cleanup. • The spilled cleanup debris must be treated as a hazardous waste following the above-mentioned disposal protocols. • Judicious usage of various other methods such as using barricade tapes, latex or nitrile gloves, usage of proper respirators (preferably N100 type), wet wipes, walk off mats, sealable bags and tight containers is considered as good laboratory practice while handling nanomaterials, and if possible, a spill kit should be prepared containing all the above-mentioned materials and made available in all such places where nanomaterials are being used or prepared.

automobiles to health care, defense to space, energy to hygiene, and many such areas that have not been imagined yet. So, it is obvious that any activity with such huge potential may lead to various questions in the field of ethical, social, and civil justice, and hence, must be addressed appropriately. At the same time, the health, safety, and environmental implications of nanomaterials are not fully known yet too. There have been some indications that nanomaterials might be harmful to humans and the environment, but more research needs to be carried out to have a clear understanding of the adverse impacts of nanotechnology. The regulatory bodies of different countries and international forums must upgrade their safety guidelines and good laboratory protocols. Indeed, this advancement of nanotechnology-based science, engineering, and technology has been a major demand for the development of sustainable nanotechnology. A huge amount of investments have led to the development of many research institutes, professional bodies, regulatory bodies, industries, and testing laboratories for creating standard operating procedures (SOPs) for measurements and evaluation. Still more funding must be diverted toward the toxicological studies of nanomaterials, thereby helping the scientists and policymakers upgrade the safety guidelines for the betterment of society.

CONFLICT OF INTERESTS

There is not any conflict of interest.

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